University of Alberta

Functional Roles of Neurotrophic Factors in the Motoneuronal Response to Axonal Injury

By

John Gordon Boyd ©

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Functional roles of neurotrophic factors in the motoneuronal response to axonal injury" submitted by J. Gordon Boyd in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

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Dedication

I would like to dedicate this thesis to my parents. Thank you for your continuing love and support, and always encouraging me to never give up while pursuing my goals. I would also like to dedicate this work to my new wife, Tracy. You truly have been the shoulder I can lean on, the rock on which I rest, and the love of my life. With you I *will* walk this path...

Abstract

The time-dependent reduction in the capacity of axotomized motoneurons to regenerate their axons is one of the major contributing factors to the poor functional recovery after peripheral nerve injury. The processes underlying this declining regenerative capacity after chronic axotomy are not well understood, and no effective treatment strategies have been developed to sustain axonal regenerative capacity over time. Neurotrophic factors, such as brain-derived neurotrophic factor (BDNF) and glial cell line-derived neurotrophic factor (GDNF) that are expressed after nerve injury are important for neuronal survival, and have been proposed to be involved in axonal regeneration. BDNF and GDNF belong to distinct families of neurotrophic factors. BDNF mediates its effects by binding to two separate cell surface receptors, trkB and p75, whereas GDNF mediates its effects via a single receptor complex consisting of a glycosyl-phophotidylinositol linked ligand binding subunit and a transmembrane signalling subunit.

A quantitative *in vivo* model of retrogradely labelling regenerated tibial motoneurons after a tibial- common peroneal nerve cross-suture has been used to characterize the effects of two neurotrophic factors on motor axonal regeneration. Continuous infusion of low dose exogenous BDNF (2 μg/day) with mini-osmotic pumps completely reversed the negative effects of chronic axotomy on motor axonal regeneration, reversed axotomy-induced somal atrophy, and promoted muscle reinnervation. The beneficial effects of low dose BDNF are attributed to the trkB receptors, due to the profound reduction in motor axonal regeneration in heterozygous trkB knockout mice. However, high dose exogenous BDNF (20 μg/day) potently inhibited motor axonal regeneration, an effect attributed to the p75 receptors because: 1) a function-blocking p75 antibody abolished the inhibitory effects of high dose BDNF, 2) there is increased motor axonal regeneration in p75 homozygous knockout mice,

3) ceramide, an intracellular signal transduction molecule downstream of p75 mimics the inhibitory effects of high dose BDNF, and 4) GDNF, which does not bind p75 shows only monophasic beneficial effects on motor axonal regeneration.

A model is presented whereby regulation of motor axonal regeneration and reinnervation of denervated muscle fibres is a complex series of events which includes a delicate balance between "growth-promoting" and "growth-inhibiting" signals mediated by neurotrophic factors.

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Table of Contents

Chapter 1: Introduction........1

1.1: General Introduction........2

1.2: Neurotrophic Factors and Their Receptors........4

1.2.1: Neurotrophin Family:........4
1.2.1.1: Introduction........4
1.2.1.2: Neurotrophin Structure........4
1.2.1.3: Neurotrophin Receptor Structure........4

1.2.2: GDNF Family........6
1.2.2.1: Introduction........6
1.2.2.2: Structure of GDNF family members........7
1.2.2.3: Structure of GDNF family receptors........7

1.2.3: Neuropoetic Cytokines........8
1.2.3.1: Introduction........8
1.2.3.2: Structure of neuropoeitic cytokines........8
1.2.3.3: Structure of neuropoetic cytokine receptors........9

1.3: *In Vitro* Resp[illegible]e of PNS Neurons to Neurotrophic Factors: Mechanisms of Survival, Differentiation, and Neurite Outgrowth........10

1.3.1: Neurotrophins........10
1.3.1.1: Summary of trk signalling events following neurotrophin binding........10

1.3.1.2: p75 signalling in response to neurotrophin stimulation........16

1.3.1.3: p75-trk interaction.....19
1.3.1.4: Neurotrophins as axon guidance molecules.....21

1.3.2: GDNF family members.....22

1.3.2.1: GDNF family member signalling via Ret.....22
1.3.2.2: Ret independent signalling.....23

1.3.3: Neuropoetic cytokines.....24

1.3.3.1: Neuropoetic cytokine signalling via gp130.....24

1.3.4: *In vitro* interactions between neurotrophic factors.....26

1.3.5: Summary.....27

1.4: Spatio-Temporal Expression of Neurotrophic Factors Relative to Peripheral Nerve Injury.....29

1.4.1: Introduction.....29

1.4.2: Motoneurons.....29
1.4.2.1: Neurotrophins and their receptors.....29
1.4.2.2: GDNF and its receptors.....31
1.4.2.3: Neuropoeitic cytokines and their receptors.....34
1.4.2.4: Intracellular signal transduction molecules.....34

1.4.3: Distal nerve stump.....35

1.4.3.1: Introduction....................35

1.4.3.2: Neurotrophins and their receptors....................36

1.4.3.3: GDNF family members and their receptors....................40

1.4.3.4: Neuropoetic cytokines and their receptors....................40

1.5: *In Vivo* Effects of Neurotrophic Factors After Peripheral Nerve Injury: Survival, Reversing the Effects of Injury, Axonal Sprouting, Regeneration, and Functional Recovery....................43

1.5.1: Survival....................43

1.5.1.1: Introduction....................43

1.5.1.2: Neurotrophins....................44

1.5.1.3: GDNF family members....................45

1.5.1.4: Neuropoetic cytokines....................46

1.5.2: Reversing the Effects of Injury....................46

1.5.3: Axonal Sprouting, Regeneration, and Functional Recovery....................48

1.5.3.1: Introduction....................48

1.5.3.2: Neurotrophins....................48

1.5.3.3: GDNF family members....................51

1.5.3.4: Neuropoeitic cytokines....................52

1.6: Conclusions and Study Objectives....................53

1.7: References....................54

Chapter 2: A Dose-Dependent Facilitation and Inhibition of Motor Axonal Regeneration by BDNF: The Role of p75 Receptors....................81

2.1: Introduction....................82

2.2: Methods....................86

2.2.1: Animal surgery and pump implantation....................86
2.2.2: Delivery of exogenous BDNF and neutralizing antibodies via Alzet mini-osmotic pumps....................87
2.2.3: Retrograde labelling of TIB motoneurons....................87
2.2.4: Animal perfusion and tissue preparation....................88
2.2.5: Enumeration of TIB motoneurons....................88
2.2.6: Histological evaluation of axonal regeneration....................88
2.2.7: Evaluation of motoneuron survival....................89
2.2.8: Statistics....................90

2.3: Results....................93

2.3.1: Fluorescent labelling of intact TIB motoneurons....................93
2.3.2: Chronic axotomy reduces TIB motor axonal regeneration....................93
2.3.3: Delivery hardware does not impair axonal regeneration....................97
2.3.4: Low dose BDNF (2 μg/day) has no effect on motor axonal regeneration after immediate nerve repair, but reverses the negative effects of chronic axotomy....................100
2.3.5: High dose BDNF (20 μg/day) inhibits motor axonal regeneration....................100
2.3.6: BDNF's enhancement and inhibition of motor axonal regeneration is dose-dependent....................108

2.3.7: Exogenous BDNF reverses soma atrophy and is not neurotoxic....................109
2.3.8: p75 mediates inhibitory effects of high dose BDNF....................114

2.4: Discussion..122
2.4.1: Low dose BDNF promotes axonal regeneration after chronic axotomy..122
2.4.2: High dose BDNF inhibits axonal regeneration..123
2.4.3: Possible mechanisms for the bimodal regulation of motor axonal regeneration by BDNF..124

2.5: Conclusion..126

2.6: References..127

Chapter 3: TrkB and p75 receptors differentially regulate motor axonal regeneration...132

3.1: Introduction..133

3.2: Methods..137

3.2.1: Experimental animals..137
3.2.2: PCR identification of trkB heterozygous knockout mice..............................137
3.2.3: Animal surgery..138
3.2.4: Evaluation of TIB motor axonal regeneration..138
3.2.4: Animal perfusion and tissue preparation..138
3.2.5: Enumeration of TIB motoneurons..139
3.2.6: Statistics..139

3.3: Results..142

3.3.1: Reduced number of TIB motoneurons in p75 (-/-), but not trkB (+/-) mice compared to wild-type controls..............................142

3.3.2: Increased motor axonal regeneration in p75 (-/-) mice compared to wild type controls....143
3.3.3: Motor axonal regeneration is initially accelerated in trkB (+/-) mice compared to wild type controls, but reaches an early plateau....146
3.3.4: Effects of p75 and trkB deletions are accentuated when normalized to contralateral intact controls....151

3.4: Discussion....154

3.4.1: Staggered motor axonal regeneration in wild type and p75 (-/-) mice, but not trkB (+/-) mice....154
3.4.2: p75 as an inhibitor of axonal growth....154
3.4.3: trkB receptors differentially regulate motor axonal regeneration....156
3.4.4: Conclusions....158

3.5: References....159

Chapter 4: The combined effects of GDNF and BDNF on motor axonal regeneration *in vivo*....165

4.1: Introduction....166

4.2: Methods....168

4.2.1: Nerve repair and pump implantation....168
4.2.2: Delivery of exogenous GDNF and BDNF via Alzet mini-osmotic pumps....168

4.2.3: Retrograde labelling of TIB motoneurons....168
4.2.4: Animal perfusion and tissue preparation....168
4.2.5: Enumeration of TIB motoneurons....171
4.2.6: Preparation of nerves for electron microscopy....171
4.2.7: Statistics....171

4.3: Results....175

4.3.1: GDNF does not promote motor axonal regeneration after immediate nerve repair....175
4.3.2: Exogenous GDNF does not show dose-dependent effects on motor axonal regeneration....176
4.3.3: GDNF and BDNF show possible synergistic effects in promoting motor axonal regeneration....181
4.3.4: GDNF and BDNF increase axonal sprouting....186
4.3.5: The beneficial effects of GDNF and BDNF are dependent on the duration of treatment....186
4.3.6: Continuous exogenous GDNF does not deter normal weight gain in experimental animals....187

4.4: Discussion....192

4.3.1: Exogenous neurotrophic factors sustain, not accelerate, motor axonal regeneration....192

4.3.2: GDNF and BDNF exert distinct effects on motor axonal regeneration....193
4.3.3: Continuous exogenous neurotrophic factor treatment is required for maximal beneficial effect....194
4.3.4: Conclusions....196

4.5: References 197

Chapter 5: Short chain ceramide analogs inhibit motor axonal regeneration *in vivo* 200

5.1: Introduction 201

5.2: Methods 203

- 5.2.1: Nerve repair and pump implantation 203
- 5.2.2: Delivery of exogenous short chain ceramide analogs and BDNF via Alzet-mini osmotic pumps 203
- 5.2.3: Retrograde labeling of TIB motoneurons 203
- 5.2.4: Animal perfusion and tissue preparation 203
- 5.2.5: Evaluation of TIB motoneuronal survival 203
- 5.2.6: Enumeration of TIB motoneurons 203
- 5.2.7: Sphingosine kinase assay 204
- 5.2.8: Statistics 204

5.3: Results 207

- 5.3.1: Short chain ceramide analogs inhibit motor axonal regeneration after immediate nerve repair, but not after chronic axotomy 207
- 5.3.2: C6 ceramide is not neurotoxic 208
- 5.3.3: Exogenous BDNF induces sphingosine kinase activity after immediate nerve repair, but not nerve repair after chronic axotomy 212

5.4: Discussion 215

5.5: References 218

Chapter 6: Effects of exogenous BDNF on the recovery of muscle and motor unit properties: a preliminary report......222

6.1: Introduction......223

6.2: Methods......227

6.2.1: Nerve repair and pump implantation......227
6.2.2: Delivery of exogenous BDNF via Alzet mini-osmotic pumps......227
6.2.3: Surgical preparation for the final experiment......227
6.2.4: Muscle and MU force recordings......228
6.2.5: Muscle fibre histochemistry......228
6.2.6: Data analysis......228
6.2.6.1: MU number......228
6.2.6.2: Muscle fibre size and number......229
6.2.6.3: Statistics......229

6.3: Results......232

6.3.1: Whole muscle properties of the reinnervated TA......232
6.3.2: Low, but not high dose BDNF improve whole muscle properties of the reinnervated TA compared to saline controls......232
6.3.3: Effects of exogenous BDNF on type, size, and number of reinnervated muscle fibres......235
6.3.4: Effects of exogenous BDNF on muscle and MU force properties......243
6.3.5: Low, but not high dose BDNF increases the number of MUs innervating the denervated TA muscle......248

6.4: Discussion......251

6.4.1: Low dose BDNF improves functional recovery.........251
6.4.2: High dose BDNF locally inhibits motor axonal regeneration, but prepares motoneurons for regeneration.........254

6.5: References.........256

Chapter 7: General Discussion.........260

7.1: Caveats and limitations of experimental model.........261

7.2: Therapeutic rationale for using neurotrophic factors to sustain motor axonal regeneration.........261

7.3: Proposed mechanism for biphasic regulation of motor axonal regeneration by exogenous BDNF.........263

7.3.1: Mechanisms of axonal regeneration.........266
7.3.2: Muscle reinnervation.........267

7.3: References.........273

List of Tables

Table 1: Summary of axotomy-induced changes in motoneurons and the distal nerve stump 42

List of Figures

Chapter 1: General Introduction

Figure 1-1: Structural and functional overlap in neurotrophic factors and their receptors......14

Figure 1-2: Temporal expression of neurotrophic factors and their receptors in axotomized motoneurons......32

Figure 1-3: Temporal expression of neurotrophic factors and their receptors in the non-neuronal cells of the distal nerve stump......37

Chapter 2: BDNF and motor axonal regeneration

Figure 2-1: Schematic representation of surgical procedures and drug delivery system......85

Figure 2-2: Effective and consistent retrograde labeling of tibial motoneurons by fluororuby and fluorogold......92

Figure 2-3: Motor axonal regeneration is reduced by chronic axotomy, but not drug delivery hardware......96

Figure 2-4: Effects of exogenous BDNF on motor axonal regeneration after both immediate nerve repair as well as nerve repair after chronic axotomy......99

Figure 2-5: Low dose exogenous BDNF reverses the negative effect of chronic axotomy but high dose exogenous BDNF reduces the number of motoneurons which regenerate their axons 102

Figure 2-6: The number of TIB axons that cross the injury site is consistent with the number of fluorescently labeled motoneurons 105

Figure 2-7: Dose-dependent facilitatory and inhibitory effects of exogenous BDNF on motor axonal regeneration after a 2 month period of chronic axotomy 107

Figure 2-8: Exogenous BDNF does not affect the number of motoneurons in the T11-L1 spinal segments 111

Figure 2-9: Quantification of the number of Nissl positive cells in the T11-L1 spinal segments demonstrates that exogenous BDNF does not affect the survival of chronically axotomized motoneurons 113

Figure 2-10: Low, but not high dose BDNF prevents motoneuronal soma atrophy after a 2 month period of chronic axotomy 116

Figure 2-11: Inhibitory effect of high dose exogenous BDNF on the number of motoneurons which regenerate axons is mediated by binding to p75 receptors 118

Figure 2-12: The p75 receptor mediates the inhibitory effect of high dose exogenous BDNF on the number of TIB motoneurons which regenerate their axons 121

Chapter 3: TrkB and p75 differentially regulate motor axonal regeneration

Figure 3-1: Identification of trkB (+/-) mice and schematic representation of surgical procedures 136

Figure 3-2: Reduced number of intact TIB motoneurons in p75 (-/-) mice compared to trkB (+/-) and wild type mice 141

Figure 3-3: Motor axonal regeneration is greater in p75 knockout mice compared to wild type controls 145

Figure 3-4: Motor axonal regeneration is initially accelerated in trkB (+/-) knockout mice compared to wild type controls, but reaches an early plateau 148

Figure 3-5: Fluororuby-labeled TIB motoneurons which regenerated axons after TIB-CP nerve repair in wild type, p75 (-/-), and trkB (+/-) mice 150

Figure 3-6: Motor axonal regeneration is differentially regulated by trkB and p75 receptors 153

Chapter 4: GDNF and BDNF in motor axonal regeneration

Figure 4-1: Schematic representation of surgical procedures 170

Figure 4-2: Exogenous GDNF has not effect on motor axonal regeneration after immediate nerve repair, but completely reverses the negative effects of chronic axotomy 174

Figure 4-3: Exogenous GDNF does not show dose-dependent effects on motor axonal regeneration 178

Figure 4-4: GDNF is more potent than BDNF in promoting motor axonal regeneration after chronic axotomy 180

Figure 4-5: The combined effects of GDNF and BDNF are greater than either factor alone 184

Figure 4-6: The ability of GDNF and BDNF to promote motor axonal regeneration is correlated with increased sprouting in the distal nerve stump 185

Figure 4-7: The ability of combined GDNF and BDNF to promote motor axonal regeneration of chronically axotomized motoneurons depends on the duration of treatment 189

Figure 4-8: Exogenous GDNF does not impede normal animal growth 191

Chapter 5: Ceramide inhibits motor axonal regeneration

Figure 5-1: Ceramide inhibits motor axonal regeneration after immediate nerve repair, but not nerve repair after chronic axotomy 206

Figure 5-2: High dose BDNF and C6 ceramide do not reduce the number of motoneurons in the TIB motoneuronal pool 211

Figure 5-3: Exogenous BDNF induces sphingosine kinase activity in a dose-dependent fashion after immediate nerve repair, but not after chronic axotomy 214

Chapter 6: BDNF promotes functional recovery of denervated muscles

Figure 6-1: Procedures for recording whole muscle and motor unit force 226

Figure 6-2: Low dose BDNF improves TA recovery 231

Figure 6-3: Muscle fibre types in intact and reinnervated TA following saline, low, or high dose BDNF treatment 234

Figure 6-4: Neither reinnervation nor exogenous BDNF significantly change the relative proportion of TA muscle fibre types 237

Figure 6-5: Low dose exogenous BDNF reduces muscle fibre CSA 239

Figure 6-6: Low dose exogenous BDNF increases the number of muscle fibres in TA muscle 242

Figure 6-7: Effects of exogenous BDNF on whole muscle properties of TA 245

Figure 6-8: Frequency distribution of TA motor unit force is shifted towards intact in BDNF treated animals compared to saline controls 247

Figure 6-9: Low dose exogenous BDNF increases the number of chronically axotomized TIB motor units which reinnervate the denervated TA muscle 250

Chapter 7: General Discussion

Figure 7-1: Possible neurotrophic factor interaction at early stages of axonal regeneration 265

Figure 7-2: Possible neurotrophic factor interaction at muscle reinnervation 272

Frequently used abbreviations:

NGF: nerve growth factor
BDNF: brain-derived neurotrophic factor
NT-3: neurotrophin-3
NT-4/5: neurotrophin-4/5
GDNF: glial cell line-derived neurotrophic factor
NTN: neurturin
PSP: persephin
ATN: artemin
CNTF: ciliary neurotrophic factor
LIF: leukaemia inhibitory factor
IL-6: interleukin-6
trk: tropomyosin receptor kinase
MAPK: mitogen associate protein kinase
erk: extracellular related kinase
PI3K: phosphatidylinositol-3 kinase
SPP: sphingosine-1-phosphate
C6-cer: C6-ceramide
C6-DHC: C6-dihydroceramide
ChAT: choline acetyltransferase
TIB: tibial nerve
CP: common peroneal nerve
TA: tibialis anterior muscle
MU: motor unit

Chapter 1: Introduction

1.1: General Introduction

Since the discovery of nerve growth factor (NGF) and its dramatic effects on the outgrowth of sympathetic fibres, many neurotrophic factors have been identified which promote neuronal survival and/or outgrowth of neurites in culture. The elucidation of the role of neurotrophic factors *in vivo* has progressively demonstrated their increasingly complex involvement in the nervous system during embryonic and postnatal development, adulthood, and following injury. In the past 10 years, our knowledge of how neurotrophic factors transduce their signal from the cell surface to the nucleus *in vitro* has exploded exponentially. Interestingly, a paradox has arisen such that our understanding of the mechanisms of signal transduction now outweigh our understanding of their functional roles *in vivo*. Several recent reviews address specific areas of neurotrophic factor research, including their roles in development (Davies, 1994; Snider, 1994; Lindsay, 1996; Conover & Yancopoulos, 1997; Chen et al., 1999; Rask, 1999;), plasticity in the central nervous system (Thoenen, 1995, 2000; Snider & Silos-Santiago, 1996; Knipper & Rylett, 1997; Lu & Figurov, 1997; Marty et al., 1997; Shieh & Ghosh, 1997; Frade & Barde, 1998; Lessmann, 1998; Takei & Nawa, 1998; Beradi & Maffei, 1999; Schuman, 1999; Schinder & Poo, 2000; Lu & Gottschalk, 2000; Beradi et al., 2000), neuronal injury (Verge et al., 1996; Ebadi et al., 1997; Fu & Gordon, 1997; Muller & Stoll, 1998; Yin et al., 1998; Terenghi et al., 1999), and signal transduction mechanisms (Segal & Greenberg, 1996; Bredesen & Rabizadeh, 1997; Kaplan & Miller, 1997, 2000; Barker, 1998; Casaccia-Bonnefil et al., 1998; 1999a,b; Chao et al., 1998; Dobrowsky & Carter, 1998; Miller & Kaplan, 1998; Friedman & Greene, 1999; Klesse & Parada, 1999; Barrett, 2000; Yano & Chao, 2000). The purpose of this introduction is to provide a comprehensive overview of neurotrophic factors and their receptors in the mammalian peripheral nervous system, with a specific focus on providing functional significance to what is known about the mechanisms by which neurotrophic factors exert their effects in the context of the motoneuronal response to injury, including survival, growth, and axonal regeneration.

Three separate and distinct families of neurotrophic factors will be compared: neurotrophins, GDNF family neurotrophic factors, and neuropoetic cytokines, based on molecular structure and receptor interactions, patterns of expression after peripheral nerve injury, and cellular effects, both *in vitro* and *in vivo*. In addition, a brief overview of the intracellular signal transduction pathways that are activated in response to these factors will provide insight into the considerable overlap, yet functional distinctiveness in cellular responses to these neurotrophic factors.

1.2: NEUROTROPHIC FACTORS AND THEIR RECEPTORS:

1.2.1: Neurotrophin family

1.2.1.1: Introduction

The neurotrophin family of neurotrophic factors is a family of structurally and functionally related peptides which mediate potent survival and differentiation effects on a wide variety of neuronal populations in the central and peripheral nervous systems. NGF, the prototypical member of the neurotrophin family, was isolated, purified and identified as a diffusible factor which promoted the survival and neurite outgrowth of sympathetic and sensory neurons both *in vitro* and *in vivo* in the classic experiments by Viktor Hamburger and Rita Levi-Montalcini almost half a century ago (Levi-Montalcini & Hamburger, 1953). Decades later, a second molecule belonging to this family was purified and cloned, brain-derived neurotrophic factor (BDNF; Barde et al., 1982). With recent advances in cloning and molecular biology, the neurotrophin family now consists of 4 members in mammals, NGF, brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Two additional members, NT-6 and NT-7 have been identified and cloned in fish, but mammalian homologs have not yet been found (Gotz et al., 1994; Lai et al., 1998).

1.2.1.2: Neurotrophin structure

Neurotrophins exist and are biologically active as non-covalent homodimers due to highly conserved structural features, including a cysteine knot motif which confers considerable interaction between each molecule of the homodimer (reviewed in McDonald & Chao, 1995; Yano & Chao, 2000). Each molecule of the homodimer consists primarily of two pairs of anti-parallel β strands which give the monomers their characteristic flat, elongated, and slightly asymmetrical shape (McDonald and Blundell, 1991). The β strands are connected by 3 short loops which are known to be highly flexible and are the regions in the neurotrophin structure where amino acid differences occur between different members of the neurotrophin family (Yano & Chao, 2000). There is an approximate 50% sequence homology between different members of the neurotrophin family (Ibid).

1.2.1.3: Neurotrophin receptor structure

Neurotrophins are unique among neurotrophic factors in that they mediate their effects by binding to two classes of receptors, the tropomyosin receptor kinase (trk) family of receptor tyrosine kinases, and a member of the tumour necrosis factor (TNF)-α family of receptors, p75 (Figure 1-1, reviewed in Yano & Chao, 2000). The p75 receptors binds all neurotrophins with similar affinity, but with different kinetics (Rodriguez-Tebar et al., 1992). In contrast, the trk family of receptors are more specific: NGF binds to trkA, BDNF and NT-4/5 bind to trkB, and NT-3 binds trkC, and trkA, albeit to a lesser extent. In addition to the full length transmembrane trk receptors, in the case of trkB and trkC, multiple truncated forms exist, which either lack an intracellular tyrosine kinase domain or posses small inserts in the intracellular tyrosine kinase domain (Barbacid, 1994). The function of these truncated receptors remains largely unknown, but may exist to either sequester neurotrophins, thus limiting binding to full length receptors in a dominant-negative fashion, or serve to cluster neurotrophins, and "present" them to their full length counterparts (Barbacid, 1994; Eide et al.,1996; Fryer et al., 1997). As there is considerable homology in the extracellular domains of the three trk receptors (~50%), it is remarkable that such selectivity exists in distinguishing individual neurotrophins (Chao, 1992). Two separate binding sites for the neurotrophins have been identified on the extracellular domains of the trk receptors (Figure 1-1): 1) a leucine-rich motif flanked by two cysteine clusters, and 2) the second of two immunoglobulin (Ig)-like domains which exists close to the cell membrane (Schneider & Schweiger, 1991). The functional significance of the leucine rich motif is poorly understood, but point mutation analysis has revealed that the latter binding site (i.e. the second Ig domain) is important in neurotrophin binding and receptor activation, as well as conferring specificity between neurotrophin ligands (Perez et al., 1995; Urfer et al., 1995).

Interestingly, in addition to binding its ligands BDNF and NT-4/5, the trkB extracellular domain has been shown to participate in cellular adhesion. *In vitro* studies using NIH 3T3 cells stably transfected with the trkB receptor have demonstrated that trkB, together with cadherin, can mediate cellular adhesion (Zhou et al., 1997). In addition, these studies showed that trkB was colocalized with the cell adhesion molecules, cadherin and cateinin, at cell-cell contact sites. The ability of the extracellular domain of trkB receptors to take part in cellular adhesion may suggest a novel and enticing role for the truncated trkB, and possibly trkC receptors in cellular mobility and motility (Zhou et al., 1997).

The structural features of the p75 receptor which confer neurotrophin binding are substantially different from the trk family of receptors (reviewed in Barker, 1998). Briefly, the extracellular domain of the p75 receptor consists of cysteine rich domains tandemly arranged, the distinguishing feature of the TNF receptor family (Figure 1-1; Smith et al., 1994; Baker & Reddy, 1996). Neurotrophins most likely interact with this ligand-binding domain on the p75 receptor via conserved positively charged residues. Targeted disruption of these positively-charged clusters abolishes neurotrophin binding to p75, but binding to their cognate trk receptors in trk-expressing fibroblasts, and survival promoting ability in isolated chick DRG neurons remains unaffected (Rydén et al., 1995).

The original nomenclature of trk and p75 receptors conferring high and low affinity neurotrophin binding has been recently been re-evaluated, based on experiments that both p75 and trkA receptors co-operate to form high affinity binding sites for NGF (reviewed in Yano & Chao, 2000). The differences in affinity can be attributed, in part, to substantial variations in binding kinetics. For example, NGF binds to trkA with relatively slow on- and off rates, but p75 associates and dissociates from NGF much faster (Mahadeo et al., 1994). The net result of this accelerated on- off- kinetic is an increase in the total amount of NGF that can bind trkA (Barker & Shooter, 1994; Mahadeo et al., 1994).

1.2.2: Glial cell-lined derived neurotrophic factor (GDNF) family

1.2.2.1: Introduction

The GDNF family of neurotrophic factors consists of 4 members, GDNF, neurturin (NTN), persephin (PSP), and artemin (ART; reviewed in Saarma & Sariola, 1999). Relative to the neurotrophins, the GDNF family of neurotrophic factors have only been recently discovered. GDNF was originally described as a potent survival factor for mesencephalic domaminergic cells, as well as motoneurons (Lin et al., 1993, 1994; Oppenheim et al., 1995; Yan et al., 1995). Three years later, the second member of this family, NTN, was identified and cloned and found to promote the survival of rat sympathetic neurons (Kotzbauer et al., 1996). The remaining members, namely PSP and ART, were identified shortly thereafter and found to have similar survival promoting activities on sympathetic neurons *in vitro* (Baloh et al., 1998; Milbrandt et al., 1998) .

1.2.2.2: Structure of GDNF family neurotrophic factors

Like the neurotrophins, members of the GDNF family are typical secretory proteins, but differ from the neurotrophins in 2 main ways. First, members of the GDNF family contain 7 cysteine residues in the same relative spacing as members of the large transforming growth factor (TGF)-β superfamily (Saarma & Sariola, 1999). Secondly, GDNF is N-glycosylated at two amino acid residues, a chemical property which is rarely found on peptide growth factors. GDNF family members share structural similarities with the neurotrophins, such as possessing a cysteine knot, which allows homodimerization, a confirmation which confers biological activity. In contrast to neurotrophin homodimers which are held together by only hydrophobic interactions, GDNF family homodimerization is also supported an additional interchain disulphide bond (Figure 1-1; see above; Yano & Chao, 2000). However, this interchain disulphide bond is not necessary to confer biological activity (Hui et al., 1999).

The second GDNF-family molecule to be discovered was NTN, which shares 42% homology with GDNF (Kotzbauer et al., 1996). The strong similarities between GDNF and NTN formed the basis for a new subfamily within the TGF-β superfamily of growth factors. PSP and ART show similar homology to GDNF, 40% and 36%, respectively (Baloh et al., 1998).

1.2.2.3: Structure of GDNF family receptors

GDNF family members exert their effects via a receptor complex which consists of a high affinity ligand binding subunit [GDNF family receptor (GFR)-α], and subsequent coupling to a common signal transduction subunit (ret; Figure 1-1). Specificity is conferred by the high affinity ligand binding subunits. GDNF binds GFR-α1, NTN binds to GFR α2, PSP binds to GFR α3, and ART binds to GFR α4, although some overlap does occur (reviewed in Saarma &Sariola, 1999). The GFR-α receptors are anchored to the plasma membrane via a glycosyl-phophotidylinositol (GPI) linkage (Figure 1-1). Interestingly, although it was originally believed that GFRα proteins functioned solely to present ret with GDNF-family molecules, it has been recently suggested that these receptors possess an intrinsic signalling capacity (Saarma & Sariola, 1999; see below *Ret-independent signalling*). Several research groups simultaneously identified ret as the signal transducing component of GDNF family member receptors (Durbec et al., 1996; Jing et al., 1996; Treanor et al., 1996; Trupp et al., 1996; Vega et al., 1996). The ret receptor shares similarities with the trk family of receptors for the neurotrophins. For example, ret is a transmembrane receptor tyrosine kinase and, like trk, was first identified as a proto-oncogene (Takahashi et al., 1985). Moreover, ret has two cadherin-like repeats in the extracellular domain, which may confer similar cell adhesion properties as described above for trkB (Figure 1-1). Thus, in light of the structural similarities between the neurotrophin and GDNF family of neurotrophic factors, and their respective receptors, functional overlap of these two families of neurotrophic factors may be predicted in the nervous system.

1.2.3: Neuropoeitic cytokines

1. Introduction

Cytokines are a large family of pleiotropic glycoprotein molecules which mediate a wide variety of biological activities associated with the induction of immune and inflammatory responses, such as initiation of both the cellular and humoral immune reactions, regulation of hematopoiesis, control of cellular proliferation and differentiation, and induction of wound healing (reviewed in Oppenheim & Saklatvata, 1993). The neuropoeitic cytokine family generally refers to molecules of the interleukin (IL)-6 family, which share a common signal transducing receptor subunit termed glycoprotein (gp) 130, and include IL-6, IL-11, leukaemia inhibitory factor (LIF), oncostatin-M (OSM), ciliary neurotrophic factor (CNTF), and cardiotrophin (CT)-1 (Heinrich et al., 1998). Since the first identification of the effect of LIF in changing the normal cardiac sympathetic noradrenergic innervation to cholinergic (Yamamori et al., 1989), a wide variety of neuronal responses have been ascribed for members of this family, including neuronal survival, differentiation, and neurite outgrowth, which will be described in detail below.

1.2.3.2: Structure of neuropoeitic cytokines

The neuropoeitic cytokines belong to a larger family of long-chain α-helix bundle cytokines, which also includes erythropoeitin, granulocyte colony stimulating factor, IL-12, growth hormone, prolactin, IL-10, interferon-α/β, and leptin (Nikola, 1994). Neuropoeitic cytokines are secretory proteins with amino terminal signal peptides, with the exception of CT-1 and CNTF which are only released upon cellular damage (Stockli et al., 1989). Unlike the neurotrophins and the GDNF-family of neurotrophic factors, the neuropoeitic cytokines are not biologically active as homodimers, but as single, four-α-helix bundle proteins (Figure 1-1; Heinrich et al., 1998). Like GDNF, certain members of the neuropoeitic cytokine family, namely IL-6, CT-1, and OSM, have N-glycosylation sites, the function of which is unknown. Neuropoetic cytokines bind to their receptors via three distinct binding sites (reviewed in Simpson et al., 1997).

1.2.3.3: Structure of neuropoeitic cytokine receptors

All members of the neuropoetic cytokine family signal via recruiting a common signal transduction receptor subunit, termed gp130 (Simpson et al., 1997; Heinrich et al., 1998; Bravo & Heath, 1999). Like the GDNF family members which do not bind ret directly (Figure 1-1), many neuropoetic cytokines do not activate gp130 directly, but first bind to specific ligand binding domains. IL-6 binds the IL-6 receptor (IL-6R), LIF binds the LIF receptor (LIFR), CNTF binds the CNTF receptor (CNTFR), OSM binds both LIFR and the OSM receptor (OSMR) (Simpson et al., 1997; Heinrich et al., 1998; Bravo & Heath, 2000). The ligand binding receptor for CT-1 remains to be identified. Together with gp130, these receptors belong to the cytokine receptor class 1 family, defined by at least 1 cytokine-binding module and at least one extracellular immunoglobulin-like domain (Yawata et al., 1993). Members of this family have one transmembrane domain, except CNTFR, which like GFR-α receptors, is a GPI-linked receptor (Figure 1-1).

1.3: *IN VITRO* RESPONSE OF PNS NEURONS TO NEUROTROPHIC FACTORS: MECHANISMS OF SURVIVAL, DIFFERENTIATION, AND NEURITE OUTGROWTH

1.3.1: Neurotrophins

1.3.1.1: Summary of trk signalling events following neurotrophin binding

The events following neurotrophins binding to their respective receptors have been extensively characterized *in vitro*, and have been the subject of many recent and extensive reviews (Segal & Greenberg, 1996; Bredesen & Rabizadeh, 1997; Kaplan & Miller, 1997, 2000; Barker, 1998; Casaccia-Bonnefil et al., 1998; 1999a,b; Chao et al., 1998; Dobrowsky & Carter, 1998; Miller & Kaplan, 1998; Friedman & Greene, 1999; Klesse & Parada, 1999; Barrett, 2000; Yano & Chao, 2000) and will be summarized briefly here.

Much of what is known about the intracellular events resulting from neurotrophins binding to trk receptors stems from research on the response of NGF binding to trkA on PC12 cells. PC 12 cells are derived from a rat pheochromocytoma cell line, express trkA and p75 receptors, and respond to NGF by differentiating into cells that resemble adult sympathetic neurons (Tischler and Greene, 1975; Greene & Tischler, 1976). Neurotrophin binding to trk receptors induces receptor dimerization and autophosphorylation of intracellular tyrosine residues of the "activation loop", and subsequent phosphorylation of specific tyrosine residues that serve as specific docking sites for important adaptor proteins which initiate intracellular signalling events (Grob et al., 1985; Kaplan et al., 1991a,b; Jing et al., 1992; reviewed in Kleese & Parada, 1999 and Yano & Chao; 2000). Many of these adaptor proteins contain src-homology-2 (SH2) or phosphotyrosine binding (PTB) domains (Figure 1-1; van der Geer et al., 1995; Borg and Margolis, 1998).

On the trkA molecule, one of these tyrosine residues that is phosphorylated upon receptor dimerization is Y490, located in the juxtamembrane region of the intracellular domain of the receptor, and is responsible for the NGF-induced trkA association with both the Shc and fibroblast growth factor receptor substrate (FRS)-2 adaptor proteins (Obermeier et al., 1993; Stephens et al., 1994; Dikic et al., 1995; Meakin et al., 1999; reviewed in Segal & Greenberg, 1996; Friedman & Greene, 1999). Y484 and Y485 are the homologous tyrosine residues on the trkB and trkC molecules, respectively (Guiton et al., 1995; McCarty & Feinstein, 1997). In addition, Y785, at the extreme C-terminal region of trkA and trkB receptors, and Y789 at the extreme C-terminus of trkC, when phosphorylated are the likely binding sites of the SH2 containing enzyme phospholipase-C (PLC)-γ (Obermeier et al., 1993; Loeb et al., 1994; Guiton et al., 1995), as well as the Csk homologous kinase (CHK; Yamashita et al., 1999). In addition to the Shc and PLC-γ binding sites, a highly conserved region of trk receptors is the "activation loop" located within the catalytic domain (Cunningham & Greene, 1998). It has been suggested that phosphorylation of the tyrosine residues in this region induce conformational changes within the catalytic domain, thus allowing greater interaction with downstream substrates (McCarty & Feinstein, 1997). Another site on the trkA molecule that is important in mediating downstream events is a lysine-phenylalanine-glycine (KFG) sequence located in the juxtamembrane region which stimulates the phosphorylation of suc1-associated neurotrophic factor-induced tyrosine phosphorylated target (SNT; reviewed in Kaplan & Miller, 1997; Friedman & Greene, 1997).

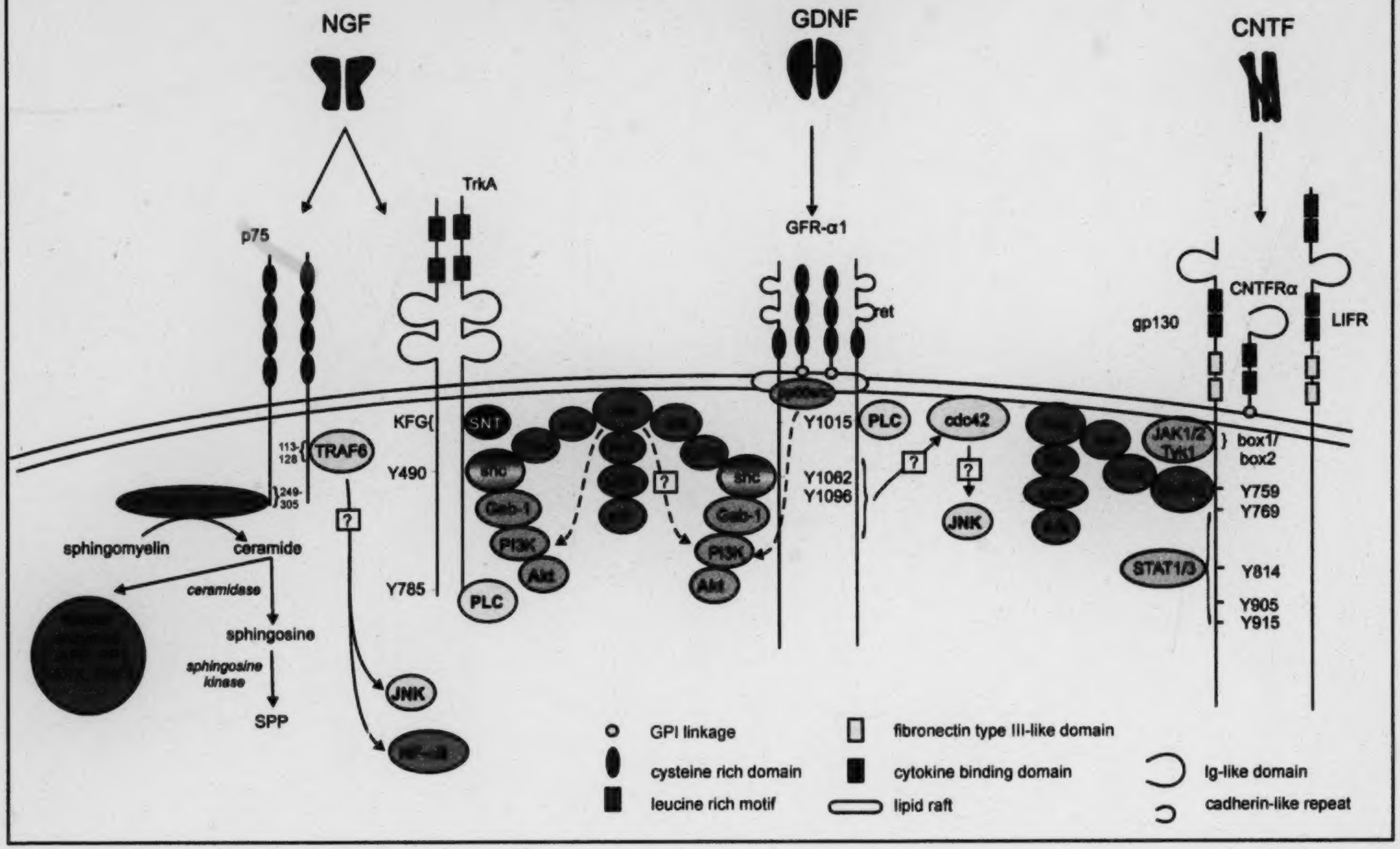
The downstream events following trk autophosphorylation and recruitment of adaptor molecules have been extensively characterized *in vitro*, not only in PC 12 cells, but primary sympathetic and sensory dorsal root ganglion neurons as well (Segal & Greenberg, 1996; Kaplan & Miller, 1997; 2000; Friedman & Greene, 1999; Klesse & Parada, 1999). A recurring theme in the trk downstream signalling pathways is the remarkable functional redundancy found in the pathways, with involvement of many different adaptor molecules, eventually leading to only a small number of functional outcomes (Figure 1-1). In brief, phosphorylation of Shc at Y490 leads to its interaction with Grb2-SOS complexes, thus activating the small G-protein p21ras (Figure 1-1). Ras can also be activated by other upstream pathways including FRS-2/SHP2/Grb/SOS, Crk/SOS, CHK, rAPS/Grb/SOS and SH2-B/Grb/SOS (Friedman & Greene, 1999; Kaplan & Miller, 2000). Activated ras initiates the extracellular related kinase (erk) pathway, also known as the mitogen associated protein kinase (MAPK) pathway. This intracellular cascade is characterized by a series of phosphorylation events which include the serine-threonine kinase raf, the tyrosine-threonine kinase map/erk kinase (MEK), and the serine-threonine kinase erk (Figure 1-1). These elements in the ras/erk pathway have been demonstrated to be necessary and sufficient to mediate the differentiation, but not survival, of PC12 cells in response to NGF stimulation (Klesse et al., 1999). In contrast, blocking ras, but not raf, MEK, or erk prevents the survival promoting effects of NGF primary sensory and sympathetic neurons (Klesse & Parada, 1998). In another study, pharmacological inhibition of MEK with PD 98059 decreased sympathetic neuron survival in the presence of constitutively activated ras by only 20% (Mazzoni et al., 1999). This suggests that, although elements of the erk pathway downstream of ras are essential in mediating the differentiation response to NGF in PC12 cells, ras can mediate survival by multiple pathways in post-mitotic differentiated primary sensory and sympathetic neurons

The activation of phophotidylinositol-3 kinase (PI3K; Figure 1-1) is central to the survival promoting effects of NGF on PC12 cells, as well as primary sympathetic and sensory neurons. It has been demonstrated that activation of PI3K is dependent on the Shc binding site of trkA in PC12 cells (Baxter et al., 1995), and that activation of the PI3K pathway may require ras activation (Klesse & Parada, 1998; Mazzoni et al., 1999). It has been suggested that the association and activation of PI3K are mediated by additional adaptor proteins, such as the insulin-receptor substrate (IRS)-1 and 2, and/or the Grb-associated binder (Gab)-1 proteins (Figure 1-1; reviewed in Friedman & Greene, 1999). The downstream target of PI3K that is important for mediating the survival effects appears to be the serine-threonine kinase Akt (Segal & Greenberg, 1996; Kaplan & Miller, 1997; Friedman & Greene, 1999; Klesse & Parada, 1999).

How does activation of the ras pathway promote the neuronal survival? *In vitro*, sympathetic neurons undergo apoptosis 24-48 hours after NGF-withdrawal, and this programmed cell death is dependent on activation of the p53 pro-apoptotic pathway that involves c-jun amino-terminal kinase (JNK), the tumour suppressor protein p53, and Bax (Aloyz et al., 1998; reviewed in Kaplan & Miller, 2000). Sympathetic neurons that have been transfected with constitutively activated ras inhibited the increases in c-jun, p53, and Bax that typically follow NGF withdrawal (Mazzoni et al., 1999). Moreover, reducing endogenous ras activity augmented

Figure 1-1: Structural and functional overlap in neurotrophic factors and their receptors. Schematic representation of prototypical members of three families of neurotrophic factors; NGF-neurotrophin family, GDNF-GDNF family members, CNTF-neuropoetic cytokines. Although there are many adaptor proteins involved in linking neurotrophic factors and their receptors to intracellular signal transduction pathways, only key overlapping and distinct pathways are shown for simplicity (see text for details).

Figure 1-1: Overview of structural and functional similarities between members of 3 different families of neurotrophic factors and their receptors


NGF
GDNF
CNTF
p75
TrkA
GFR-α1
ret
gp130
CNTFRα
LIFR
113-128
249-305
TRAF6
?
KFG
SNT
Y490
Y785
snc
Gab-1
PI3K
Akt
PLC
Y1015
Y1062
Y1096
cdc42
JNK
NF-κB
JAK1/2 Tyk1
box1/ box2
Y759
Y769
STAT1/3
Y814
Y905
Y915
sphingomyelin
ceramide
ceramidase
sphingosine
sphingosine kinase
SPP
GPI linkage
cysteine rich domain
leucine rich motif
fibronectin type III-like domain
cytokine binding domain
lipid raft
Ig-like domain
cadherin-like repeat

the increase in expression of c-jun, p53, and Bax following NGF withdrawal (Ibid). Thus, in sympathetic neurons, ras promotes survival by suppressing the p53 pro-apoptotic pathway (reviewed in Kaplan & Miller, 2000). It is important to note that it is the kinetics of JNK expression that are important in determining cellular fate, as transient JNK induction is more associated with cellular proliferation, whereas sustained activation leads to apoptosis (Chen et al., 1996)

Two additional signalling events which occur following NGF activation of trkA are the activation of SNT and PLC-γ (Figure 1-1). As mentioned above, SNT activation is dependent on the presence of the conserved KFG sequence in the juxtamembrane region of the trkA receptor. SNT appears to be involved in neuritogenesis, as targeted deletion of this KFG region results in a loss of SNT phosphorylation, and a failure to demonstrate neurite outgrowth (Peng et al., 1995). Phosphorylated PLC-γ cleaves phophotidylinositol 4,5 bisphosphate into two intracellular messengers, inositol triphosphate (IP_3) and diacylglycerol (DAG). The generation of IP3 leads to a rise in intracellular calcium via binding to its receptor on the endoplasmic reticulum, whereas DAG stimulates protein kinase C (PKC) activation (Berringer et al., 1993; Berridge, 1993). It is possible that the increase in intracellular calcium may serve to induce neurotrophin stimulation in an autocrine manner, as it has been demonstrated that calcium influx can trigger BDNF transcription via phosphorylation of a calcium response element binding protein (CREB) dependent mechansim (Tao et al,. 1998). Although it is known that the activation of PLC-γ can be an alternative pathway for the activation of ras, the mechanism by which this occurs is unknown (Segal & Greenberg, 1996).

In summary, the functional outcomes of neurotrophin binding to trk receptors on PC 12 cells, or isolated primary sympathetic and sensory neurons *in vitro* can be divided into 3 major categories, survival, differentiation, and neuritogenesis, and are very roughly associated with activation of the PI3K-Akt, ras-erk, and SNT pathways, respectively; however, considerable overlap does occur. Although most experiments have focussed on the interactions between NGF and trkA on PC12 cells, sympathetic, and sensory neurons, due to the high homology between the neurotrophins and their receptors, it is anticipated that these events can be generalized to other members of the neurotrophin family. In fact, it was recently demonstrated that in sympathetic neurons stably expressing trkB, phosphorylated trkB stimulated signaling proteins and induced survival and growth in a manner similar to trkA (Atwal et al., 2000). Analogous to trkA, 1) mutations at the Shc binding site reduced the ability of BDNF to promote survival and growth, whereas mutations at the PLC-γ were with little effect, 2) survival was dependent primarily on PI3 kinase, and to a lesser extent MEK, and 3) growth depended on both MEK and PI3 kinase activity (Ibid). The importance of PI3 kinase in mediating the survival promoting effects of BDNF may be extended to motoneurons as well. BDNF activation of PI3, but not erk kinase promoted the survival of isolated embryonic chick motoneurons (Dolcet et al., 1999). However, elements of the erk pathway are important in mediating the survival promoting effects of neurotrophic factors in mammalian motoneurons, as embryonic motoneurons isolated from mice carrying a homozygous deletion of B-raf no longer survive in response to BDNF, GDNF, or CNTF (Wiese et al., 2001).

1.3.1.2: p75 signalling in response to neurotrophin stimulation

In contrast to the well delineated pathways and their functional significance that have been identified for trk signal transduction, the physiological role of the p75 receptor has been more difficult to define. Barker (1998) delineated the effects of the p75 receptor into two broad categories: 1) p75 signals independently to induce apoptosis, in a manner similar to other members of the TNF-α family of receptors, and 2) p75 functionally cooperates with trk receptors to either a) enhance, or b) reduce neurotrophin-mediated trk activation. This section will focus on p75 independent signalling, and p75-trk interactions will be discussed in the following section.

There are several salient features to p75 receptor signalling which have been the subject of many recent reviews and will only be highlighted here (Bresden & Rabizadeh, 1997; Kaplan & Miller, 1997,2000; Barker et al., 1998; Dobrowsky & Carter, 1998; Cassicca-Bonnefil et al., 1998,1999a,b; Chao et al., 1998; Friedman & Greene; 1999; Barrett, 2000). Generally speaking, a cohesive picture of p75 signalling has been difficult because the effects of neurotrophin binding to p75 depend greatly on cell type, developmental stage, and ligand.

One of the well documented downstream events following neurotrophin binding to p75 receptors is the activation of sphingomyelinases and induction of sphingomyelin hydrolysis, leading to the production of ceramide (Figure 1-1; reviewed in Dobrowsky & Carter, 1998). Ceramide is a lipid second messenger that is also produced as a result of TNF-α family members binding to their cognate receptors, and event intimately linked with the pro-apoptotic functions of these proteins (reviewed in Perry et al., 1998). It is not known how ligand binding to p75 leads to sphingomyelinase activation, but appears to required a sequence within amino acids 249-305 of the cytoplasmic tail (Figure 1-1), as targeted deletion of this sequence abolishes sphingomyelinase activation, but not NGF binding or expression in the membrane (Dobrowsky et al., 1995).

In the nervous system, a wide variety of responses have been attributed to the generation of ceramide. These responses include the induction of JNK in oligodendrocytes leading to cell death (Casaccia-Bonnefil et al., 1996), induction of NF-κB in Schwann cells (Carter et al., 1996), promoting survival of isolated sensory neurons (Ping & Barrett, 1998), promoting neurite outgrowth of cultured hippocampal neurons (Brann et al., 1999), inhibiting neurite outgrowth of sympathetic neurons (Posse de Chaves et al., 1997), and modulating trkA signalling in PC12 cells (MacPhee & Barker, 1997, 1999).

To explain the diverse cellular responses to ceramide, Perry & Hannun (1998) identified several factors important in determining a cell's response to ceramide, such as the number of downstream effector enzymes which are activated by ceramide, and the activities of various enzymes involved in ceramide metabolism. The downstream effector enzymes activated by ceramide include ceramide activated protein phosphatase (CAPP; a member of the 2A class of protein phosphatases), protein phosphatase-1 (PP1), ceramide activated protein kinase (CAPK), and protein kinase C-ζ (Figure 1-1; reviewed in Perry & Hannun, 1998). The role of these effector enzymes in mediating the effects of ceramide in neurons remains to be examined. However, experiments in other non-mammalian, non-neuronal cell types suggest there are many levels at which ceramide effector enzymes may interact with known neuronal signalling pathways, contributing to our difficulty in isolating a physiological for ceramide in neurons. For example, in transformed human epithelial cells, both CAPP and PP1 have been demonstrated to activate c-jun (Reyes et al., 1996). Also, in *Drosophila,* CAPK has been demonstrated to be the kinase suppressor of ras (Zhang et al., 1997) and directly involved in the activation of raf, and thus the erk pathway (Yao et al., 1995).

The second factor that may determine a cell's response to ceramide is the presence of enzymes involved in ceramide metabolism into biologically active metabolites, namely ceramidase and sphingosine kinase. Ceramidase cleaves ceramide to form sphingosine and a free fatty acid, the sphingosine can be phosphorylated by sphingosine kinase to form the biologically active sphigosine-1-phosphate (Figure 1-1; SPP; Spiegel et al., 1996). SPP has been shown to reverse the pro-apoptotic functions of ceramide, thus suggesting that cellular fate may depend on a balance between ceramide and SPP (Cuvillier et al., 1996), and further, that regulation of the enzymes involved in ceramide metabolism may serve as important sites for regulation of cellular survival. Indeed, trkA activation via NGF induces a biphasic increase in sphingosine kinase activity in PC12 cells, and treatment with SPP protected these cells from apoptosis induced by serum withdrawal (Edsall et al., 1997). Furthermore, not only does SPP act as an intracellular messenger, but it also mediates ligand specific effects via binding to the EDG-1 family of G-protein coupled receptors (Spiegel, 2000).

Recent experiments suggest that neurotrophin binding to p75 may activate pathways distinct from the induction of sphingomyelinase activity. Other members of the TNF family of receptors interact with TNF receptor associated factors (TRAFs) via a conserved intracellular element termed the "death domain", to modulate apoptosis, as well as JNK and NF-κB activity (Rothe et al., 1995; Song et al., 1997). The cytoplasmic juxtamembrane region tail of p75 has been shown to associate with TRAF6 in a ligand-dependent manner, and requires sequences between residues 113 and 128 (Figure 1-1; Khursigara et al., 1999). In the same study, a dominant negative form of TRAF6 was shown to inhibit the p75-dependent NF-κB nuclear translocation in response to NGF treatment. Furthermore, several other adaptor proteins have been shown to interact with the p75 receptor in GST pulldown assays, such as neurotrophin receptor interacting factor (NRIF), SC-1, and neurotrophin receptor-interacting melanoma antigen gene homolog (NRAGE) (reviewed in Kaplan & Miller, 2000). These adaptor proteins are associated with apoptosis (NRIF) and cell cycle arrest (SC-1 and NRAGE), but their role in axonal growth or regeneration remains undetermined.

1.3.1.3: p75-trk interaction

As described above, trkA receptors require co-expression of p75 for formation of high affinity binding states, and evidence from p75 homozygous knockout mice demonstrate that co-expression of these receptors are required for maximal survival of neurons during periods of development when neurons are competing for limiting amounts of neurotrophins. For example, in p75 knockout mice, there is significantly more developmental cell death in sensory (Lee et al., 1992, 1994; Davies et al., 1993), and motoneuronal (Ferri et al., 1998) cell populations. The enhanced cell death may be explained by a rightward shift in the dose-response curves for the amount of neurotrophin required to elicit maximal survival responses. Weise et al (1999) found that embryonic motoneurons isolated from p75 deficient mice required 5 times more BDNF to elicit maximal survival responses than wild-type control motoneurons, consistent with higher concentrations of NGF to promote survival of sympathetic neurons in p75 knockout mice (Lee et al., 1994).

Two models have been proposed to explain the collaborative interaction between p75 and trk receptors (reviewed in Cassicca-Bonnefil et al., 1999). Briefly, the first model suggests that the high affinity state is a result of the p75 receptor "presenting" trk receptors with bound neurotrophin (Barker & Shooter, 1994), whereas the second model proposes that p75 and trk receptors are able to demonstrate neurtrophin-independent association, in other words, p75 induces a conformational change in trk receptors which confers high affinity binding (Chao & Hempstead, 1995). The latter is supported by experiments which demonstrate that high molecular weight receptor complexes that bind NGF can be recognized by both p75 and trkA antibodies (Ross et al., 1998). The specific nature of p75-trk collaborative interaction remains to be elucidated.

There are several recent lines of evidence from both PC 12 cells and sympathetic neurons that suggest that activation of the p75 receptor antagonizes trk signal activation

(McPhee & Barker, 1997; Aloyz et al., 1998; Kohn et al., 1999). In PC12 cells expressing p75 and trkA, BDNF-mediated activation of p75 reduced the ability of NGF to induce tyrosine phosphorylation of trkA receptors in a dose-dependent manner (McPhee & Barker, 1997). The reduction in trkA phosphotyrosine content was associated with 1) a reduction in c-fos activation, a transcription factor normally activated in response to trkA, and 2) an increase in phosphoserine content. This effect may be mediated by the p75-induced generation of ceramide, as transient stimulation with a short-chain ceramide analog mimicked this effect (Ibid). It is not currently understood how this increase in phosphoserine content confers reduced trkA activation, however, it provides an enticing mechanism by which p75 activation can modulate trkA activity. Interestingly, although transient stimulation with short chain ceramide analogs decreased trkA responsiveness to NGF, long-term ceramide application actually enhanced trkA responsiveness to NGF (McPhee & Barker, 1999).

Further *in vitro* studies have characterized p75 antagonism of trkA activity. If sympathetic neurons are maintained at sub-optimal concentrations of NGF, BDNF activation of p75 can antagonize trkA mediated survival (Aloyz et al., 1998; Bamji et al., 1998) and axonal growth (Kimpinski et al., 1997; Kohn et al., 1999) in a dose-dependent fashion. In addition, preventing autocrine activation of p75 with a function blocking anti-p75 antibody directed towards the extracellular domain of p75 (Weskamp & Reichardt, 1991), or with an anti-BDNF antibody also increased neurite outgrowth in these cells (Kohn et al., 1999). Furthermore, sympathetic neurons isolated from p75 knockout mice show increased neurite outgrowth, and do not show an inhibitory effect on axonal growth in response to BDNF compared to wild-type control neurons (Ibid). It is possible that p75 antagonism of axonal growth, like trkA autophosphorylation, involves ceramide, as elevation of intracellular ceramide, and application of short chain ceramide analogs to the distal axons of sympathetic neurons inhibits axonal elongation *in vitro* (Posse de Chaves et al., 1997). The question as to whether ceramide inhibits axonal growth *in vivo* remains to be addressed.

In vivo experiments have also suggested a role for p75 in negatively regulating trk-mediated neurite growth. Sympathetic target organs such as the pineal gland which contain high levels of BDNF are hyperinnervated by tyrosine hydroxylase positive sympathetic axons in p75 knockout mice (Kohn et al., 1999). There is also extensive CNS sprouting of sympathetic and sensory fibres in p75 knockout mice which overexpress NGF under a glial-specific promoter (Walsh et al., 1999). This negative regulation of trk by p75 is not necessarily restricted to trkA, and may also extend to trkB, or trkC, as evidenced by increased motoneuronal survival and axonal sprouting following facial nerve crush in p75 knockout mice (Ferri et al., 1998). In addition, administration of BDNF exerts a biphasic dose-dependent effect on the survival of isolated embryonic motoneurons from wild type mice, where lower doses promote survival, but at progressively higher doses, BDNF becomes progressively less effective in mediating this effect (Weise et al., 1999). However, this decline in effectiveness is not apparent in embryonic motoneurons isolated from p75 homozygous knockout mice. It may be possible that increasing doses of BDNF shift the balance between trkB and p75 activation, thus at high doses of BDNF, p75 activation predominates and antagonizes the survival signal mediated by trkB.

The antagonism between trk and p75 can be bidirectional (reviewed in Kaplan & Miller, 1997). Not only does p75 negatively regulate trk activity, but high levels of trk were shown to inhibit p75 activation. In PC12 cells which do not express trkC, NT-3 induced significant sphingomyelin hydrolysis and the production of ceramide; presumably via p75 receptors (Dobrowsky et al., 1995). However, NGF did not induce sphingomyelin hydrolysis in these cells, perhaps because NGF signalling via trkA inhibited NGF signalling via p75, an idea supported by the fact that NGF was able to induce sphingomyelin hydrolysis in the presence of K252a, an inhibitor of trk tyrosine kinase activity (Ibid). The downstream target of activated trkA which is responsible for mediating the negative regulation of p75 is PI3 kinase, as pharmacolocial inhibition of PI3 kinase restored the ability of NGF to induce sphingomyelin hydrolysis (Bilderback et al., 2001).

1.3.1.4: Neurotrophins as axon guidance molecules

Neurotrophins are unique among neurotrophic factors in the ability to act as guidance molecules for growing growth cones (Song et al., 1997; Ming et al., 1999; Zheng et al., 2000; Tucker et al., 2001). Studies of growth cone turning responses of *Xenopus* spinal neurons have identified several molecules which can attract or repulse growth cones, and have divided these molecules into two groups (Song et al., 1997). BDNF is considered a group 1 guidance molecule based on: 1) its ability to induce turning responses in growth cones of *Xenopus* spinal neurons, 2) dependence on extracellular calcium, and 3) regulation by cAMP and PKC. In contrast, NT-3 is considered a group 2 guidance molecule because it is not dependent on extracellular calcium, and is regulated by cGMP or protein kinase G (PKG; Song et al., 1997). Until recently, the role of NGF in growth cone turning was unknown because *Xenopus* spinal neurons do not express endogenous trkA. *In vitro* experiments evaluating the turning responses of growth cones of *Xenopus* spinal neurons which have been transfected with either wild type trkA, or mutant trkA unable to activate Shc, PI3 kinase, or PLC-γ have demonstrated that NGF behaves as a group 1 guidance molecule, and coactivation of PLC-γ and PI3 kinase is essential for turning responses to a gradient of NGF (Ming et al., 1999).

The role of the neurotrophins in mammalian growth cone chemotaxis has been newly elucidated. By expressing enhanced green fluorescent protein under a neuron-specific promoter, Tucker et al. (2001) were able to observe spinal neuron growth in response to gradients of neurotrophins in real time. Chromatographic beads soaked in NGF, NT-3, BDNF, and NT-4/5 were all able to induce chemotaxis in sensory, but not motor neurons. Functional blocking antibodies to the neurotrophins abolished the turning response of both motor and sensory neurons. The authors suggest that adhesive forces between "neurotrophin dependent" sensory and "neurotrophin independent" motor axons could result in inhibition of the growth of both populations when the outgrowth of one is potently inhibited (Tucker et al., 2001). Thus specific chemotactic molecules for developing motor axons remain to be identified.

1.3.2: GDNF family members

1.3.2.1. GDNF family member signalling via Ret

Elements involved in Ret signal transduction following dimerization with GFRα receptors has been recently reviewed (Airaksinen et al., 1999) and will be summarized here to compare and contrast with elements involved in signal transduction pathways activated by other neurotrophic factor families. Upon dimerization, several key tyrosine residues on Ret become phosphorylated and serve as docking sites for intracellular adaptor proteins. Grb7 and Grb10 associate with Y905, PLC-γ with Y1015, Shc with Y1062, and Grb2 with Y1096 (Asai et al,. 1996; Borrello et al., 1996; Durick et al., 1996; Pandey et al., 1996; Arighi et al., 1997; Lorenzo et al., 1997; Ohiwa et al., 1997; Alberti et al., 1998; Xing et al., 1998; Shc and Grb2 sites shown in Figure 1-1). PLC-γ, Shc, and Grb2 also associate with phosphorylated trk receptors (see above), and may at least partially explain the functional redundancy of these two distinct families of neurotrophic factors on cellular populations which express both types of receptors. Subsequently, similar downstream signalling pathways are activated, such as the ras-erk pathway (Santoro et al., 1994; Worby et al., 1996; Soler et al., 1999, Trupp et al., 1999), PI3K (van Weering & Bos, 1997; Soler et al., 1999, Trupp et al., 1999), and PLC-γ (Borello et al., 1996, Trupp et al., 1999). The GDNF induced activation of PI3K and survival promoting effects have also been shown to involve the association of the src kinase, pp60src, with activated ret (Encinas et al., 2001).

In addition, GDNF can activate Rho/Rac-related small GTPases, such as Cdc42, thus leading to the activation of JNK (Figure 1-1; Chiariello et al., 1998). As mentioned above (see section 1.3.1.1: *Summary of trk signalling events following neurotrophin binding*), although the expression of JNK is often associated with apoptosis (Casicca-Bonefil et al., 1996; Bamji et al., 1998; Yoon et al., 1998), it is the kinetics of JNK expression that are important in determining cellular fate, as transient JNK induction is associated with cellular proliferation, whereas sustained activation leads to apoptosis (Chen et al., 1996).

In PC12 cells, activation of the ras-erk kinase pathway appears to be necessary for the survival and neurite outgrowth stimulating actions of GDNF and NTN (Creedon et al., 1997; van Weering & Bos, 1997; Xing et al., 1998). Also, PI3 kinase signalling is required for GDNF induced formation of lamellipodia, which are important for neuritogenesis (van Weering & Bos, 1997; van Weering et al., 1998). In contrast, as with exogenous BDNF (Dolcet et al., 1999), activation of PI3 kinase, but not the erk-MAPK pathway was responsible for mediating the survival promoting effects of GDNF family members on isolated embryonic motoneurons (Soler et al., 1999).

1.3.2.2: Ret-independent signalling

Saarma & Sariola (1999) identify 3 lines of evidence that GFRα receptors can signal independently of ret. First, in some tissues GFRα receptors are expressed without ligand or ret. Secondly, GDNF supports the survival of postnatal cochlear ganglion neurons, which express GFRα1, but not ret. Third, GDNF induces increases intracellular calcium levels in sensory neurons isolated from mice carrying a homozygous mutation for ret. GFRα receptors, like other GPI-linked receptors, may be associated with lipid rafts, which are detergent insoluble "microdomains" within the plasma membrane which are rich in cholesterol and sphingomyelin (Figure 1-1). Lipid rafts are considered specialized signalling organelles within the plasma membrane (reviewed in Jacobson & Dietrich, 1999). Lipid rafts are generally enriched in signalling proteins which localize to the intracellular leaflet of the plasma membrane, thus GFRα receptors may mediate intracellular signalling events such as activation of src family kinases and elevations of intracellular calcium (Airaksinen et al., 1999). This is supported by a recent studies which demonstrate that in cell lines which expresses high levels of GFRα1, but not ret, GDNF stimulation promotes cell survival, and is associated with activation of src-like kinases in detergent insoluble membrane fractions, suggesting the involvement of lipid rafts (Poteryaev et al., 1999; Trupp et al., 1999).

Interestingly, in cells that express both GFRα1 and ret, GDNF binding to GFRα1leads to ret translocation to lipid rafts as well as ret association with pp60 src, and disrupting ret's ability to associate with lipid rafts leads to attenuated cellular responses to GDNF, such as survival, as well as phosphorylation of erk and Akt (Tansey et al., 2000; Encinas et al., 2001). Thus, it is becoming clear that both ret-dependent, and ret-independent signalling depends on association with lipid rafts (Figure 1-1; Tansey et al., 2000; Encinas et al., 2001).

1.3.3: Neuropoeitic cytokines

1.3.3.1: Neuropoeitic cytokine signalling via gp130

The intracellular events involved in neuropoetic cytokine signalling have been recently reviewed and will only be briefly summarized here (Simpson et al., 1997; Heinrich et al., 1998; Turnley & Bartlett, 2000). The best described intracellular pathway that is activated in response to neuropoetic cytokine stimulation is the Janus kinase (JAK)-signal transducers and activators of transcription (STAT) pathway. This pathway is common to other cytokines, such as interferons as well as other growth factors. The first event in the induction of the JAK-STAT pathway is ligand dependent homo- or heterodimerization of signal transduction subunits such as gp130, LIFR, and OSMR. Upon ligand binding, all neuropoeitic cytokines recruit the signal transducing gp130 to the receptor complex, which can either signal alone, or in combination with either LIFR or OSMR. The signal transducing receptors, gp130, LIFR, and OSMR are all able to induce the phosphorlyation of JAKS, and thus recruit STAT proteins to the intracellular domain of the receptor complex. The exact composition and conformation of the ligand-receptor complex is ligand specific. For example, IL-6 binding to IL-6R induces gp130 homodimerization, but CNTF, LIF, and CT-1 binding to their cognate ligand binding receptors induces heterodimerization of gp130 and LIFR (Figure 1-1; Heinrich et al., 1998). As with the GDNF family of neurotrophic factors, IL-6 and CNTF first bind to specific α-receptor units which are not directly involved in the signal transduction cascade (Figure 1-1; shown as CNTFR α). A ligand binding alpha subunit has been proposed for CT-1, but so far has not been identified. Another similarity between GDNF family receptors and IL-6/CNTF receptors is that the membrane bound α-receptor subunit can be functionally replaced by soluble forms which lack the transmembrane domain and cytoplasmic domain. In contrast, LIF and OSM bind directly to their signal transduction receptors, LIFR and OSMR, respectively. Upon ligand binding and receptor homo- or heterodimerization, gp130 associated JAKs, Jak1, Jak2, and Tyk2 become activated via their association with Box1/Box2 domains of gp130, and the cytoplasmic tail of gp130 becomes phosphorylated at several phosphotyrosine residues which serve as docking sites for STAT proteins which contain SH2 binding sequences (Figure 1-1; STAT3 and STAT1). The association of STATs with gp130 can induce STAT phosphorylation and dimerization, at which time the STATs can translocate to the nucleus where they regulate transcription of target genes.

In addition to STAT activation, phosphorylated gp130 also serves as a docking site for SHP2 at Y759 (Figure 1-1). SHP2 is a tyrosine phosphotase which may form a link to the ras-erk pathway, which like the neurotrophins and GDNF family neurotrophic factors, is also activated in response to neuropoeitic cytokine stimulation (Ernst et al., 1996; Heinrich et al., 1998; Kim et al., 1999; Figure 1-1). As described above, SHP2 also serves as a adaptor protein for activation of ras via its association with Grb2 in response to neurotrophin stimulation (Klesse and Parada, 1999). Recently, Shc, the adaptor protein involved in linking trk (neurotrophin family signaling) and ret (GDNF family signaling) activation to the ras-erk, as well as the PI3K pathways (Figure 1-1), has been shown to be recruited to activated OSMR (Hermanns et al., 2000), suggesting further points of overlap between neurotrophin-, GDNF-, and neuropoeitic families of neurotrophic factors.

In contrast to the functional significance that has been attributed to the different aspects of both neurotrophin and GDNF family neurotrophic factor signal transduction, little is known about the specific role of the different elements involved in neuropoeitic cytokine signal transduction. It is known however, that these factors do promote the survival and differentiation of many different peripheral neuronal populations *in vitro*. For example, IL-6, CNTF and LIF all promote the survival of embryonic sensory and motoneurons (Hama et al., 1989; Arakawa et al., 1990; Ip et al., 1991; Sendtner et al., 1991; Martinou et al., 1992; Clatterbuck et al., 1993; Curtis et al., 1994; Hirota et al., 1996; Thier et al., 1999). Recently, exogenous CT-1 has also been shown to promote the survival of embryonic motoneurons (Pennica et al., 1996; Arce et al., 1999; Oppenheim et al., 2001). The ability of CNTF, LIF and CT-1 to promote the survival of embryonic motoneurons depends on co-expression of LIFR (Arce et al., 1999). In addition, IL-6 can promote the differentiation of PC12 cells in a manner similar to NGF (Marz et al., 1998)

Thus, although it is well established that members of the neuropoeitic cytokine family are important in mediating the survival and differentiation of different populations of peripheral neurons *in vitro*, the specific intracellular signalling mechanism(s) by which this occurs remain(s) to be determined.

1.3.4: *In Vitro* interactions between neurotrophic factors

Due to the significant overlap in adaptor proteins recruited to activated receptors and signal transduction mechanisms utilized by the 3 distinct families of neurotrophic factors outlined in the above sections (Figure 1-1), one may predict that these factors also demonstrate significant interactions in biological responses that are elicited upon application to responsive cell types. This is supported by several lines of experimental evidence which demonstrate that combinations of members of the neurotrophin family, GDNF family, and neuropoetic cytokines interact to elicit either additive or synergistic effects.

In studying the effects of BDNF, GDNF, and CNTF on embryonic motoneuron differentiation, all three neurotrophic factors promoted the expression of ChAT, as well as neurite outgrowth, albeit to varying degrees (Zurn et al., 1996). BDNF had the largest effect on ChAT activity, whereas GDNF had the largest effect on neurite outgrowth. Combinations of GDNF and BDNF had additive effects on ChAT activity, whereas GDNF and CNTF, or BDNF and CNTF had synergistic effects (Ibid). This difference may be explained in part, by the fact that the intracellular signalling pathways activated by GDNF and BDNF are largely overlapping, whereas CNTF may activate both the ras-erk and JAK-STAT pathways (Figure 1-1). This is consistent with synergistic effects of GDNF and CT-1 on promoting the survival of cultured embryonic motoneurons (Arce et al., 1998). Substantial convergence of the intracellular pathways activated by the 3 families of neurotrophic factors is also supported by the fact that although BDNF, GDNF, and CNTF all promote the survival of embryonic motoneurons *in vitro*, the ability of all three of these factors to promote survival is abolished in mice lacking B-raf (Wiese et al., 2001), an essential component in the ras-erk kinase pathway (Figure 1-1). This suggests a common "survival" signal transduction pathway that is activated by distinct families of neurotrophic factors.

There is also evidence for neurotrophic factor interaction in embryonic sensory neuron survival. The ability of IL-6 to promote the survival of embryonic DRG neurons depends on a basal level of endogenous BDNF activity, as anti-BDNF antibodies and anti-trkB antibodies reduce the ability of IL-6 to support the survival of a subpopulation of sensory neurons (Murphy et al., 2000).

1.3.5: Summary

Neurotrophins, GDNF family members, and neuropoetic cytokines display many key similarities and differences in their structure, receptor systems, intracellular signal transduction pathways, and *in vitro* biological activity. Specifically, neurotrophins and GDNF family members exist and are biologically active as homodimeric molecules, whereas the neuropoetic cytokines are long chain α-helix bundle proteins. Although the neurotrophins bind to two distinct classes of receptors, trk and p75, the trk-p75 interaction in the formation of high affinity binding sites for neurotrophins may be considered analogous to the receptor systems used by GDNF family members and neuropoetic cytokines, which include the ligand binding α-subunits and the transmembrane signal transduction subunits. Furthermore, we have highlighted both the redundant and distinct signal transduction pathways that are activated by these three families of neurotrophic factors which is consistent with the ability of these neurotrophic factors to exert similar biological effects on many neuronal populations, such as survival, differentiation, and neurite outgrowth.

In the following sections, we will provide a detailed description of the regulation of neurotrophic factors and their receptors, as well as several key signal transduction molecules, following peripheral nerve injury. We will focus on the expression of the neurotrophins, GDNF family members, and the neuropoetic cytokines, in axotomized motoneurons and in the regenerative environment of the distal nerve stump. By examining the temporal expression of these neurotrophic factors after injury, we aim to provide insight into the mechanisms by which neurotrophic factors exert their effects on axotomized motoneurons *in vivo*, such as promoting survival, regenerative sprouting, and functional recovery.

1.4: SPATIO-TEMPORAL EXPRESSION OF NEUROTROPHIC FACTORS RELATIVE TO PERIPHERAL NERVE INJURY

1.4.1: Introduction

Following a discussion of the signal transduction mechanisms and pathways activated by neurotrophic factors binding to their receptors, it is evident that not only is it important to know what neurotrophic factors are present following peripheral nerve injury, but also their 1) kinetics of expression, and 2) the relative expression of neurotrophic factors and their receptors. Thus, prior to examining the roles of neurotrophic factors in peripheral nerve injury, we will first describe the spatial and temporal regulation of neurotrophic factors and their receptors in motoneurons (Figure 1-2), and the distal nerve stump (Figure 1-3) following peripheral nerve injury.

1.4.2: Motoneurons

1.4.2.1: Neurotrophins and their receptors

Motoneuronal expression of neurotrophins after injury has been well described. NGF is not expressed by motoneurons, nor is it upregulated after injury (Funakoshi et al., 1993; Escandon et al., 1994). In contrast, BDNF is expressed at relatively low levels in intact motoneurons, and is rapidly induced after injury. As early as 8 hours after axotomy, BDNF mRNA is increased in facial motoneurons (Kobayashi et al., 1996), as detected by *in situ* hybridization (Figure 1-2A). Two days after injury, most motoneurons express high levels of BDNF mRNA (Kobayashi et al., 1996). However, this increase in BDNF gene expression is transient, as BDNF mRNA expression begins to decline at 4 days, and returns to baseline by 7 days after injury. Quantification of the serial dilution RT-PCR amplification of BDNF mRNA demonstrates a twofold increase at 8 hours, 4 fold increase at 12 hours, returning to twofold increase at 1 day, declining to 1.5 fold increase at 4 days, and returning to baseline at 7 and 14 days after injury (Figure 1-2A; Kobayashi et al., 1996). The transient nature of BDNF mRNA expression as detected by *in situ* hybridization and RT-PCR is consistent with results using RNAse protection assay on homogenized spinal cord (Funakoshi et al., 1993).

The upregulation of BDNF mRNA as detected by *in situ* hybridization, RT-PCR, and RNAse protection assay corresponds with BDNF protein expression. BDNF protein is detected as early as 1 day following facial nerve transection, reaches a peak at 7 days, and remains elevated compared to contralateral intact control motoneurons as late as 14 days post injury (Kobayashi et al., 1996).

Less is known about the kinetics of NT-3 and NT-4/5 expression after injury. Evaluation of spinal cord homogenates after sciatic nerve injury has suggested that the expression of both NT-3 and NT-4/5 in motoneurons decreases after injury (Figure 1-2A; Funakoshi et al., 1993). NT-3 mRNA shows a biphasic response: decreasing twofold 12 hours after axotomy, recovering to baseline by 3 days, but again decreasing twofold 2 weeks later (Ibid). NT-4/5 decreases slightly 6 hours after sciatic nerve injury, but returned to control levels at 12 hours (Ibid). However, it is not clear whether the mRNA detected in these studies truly reflects motoneuronal expression, or other cells present in the spinal cord, such as interneurons, astrocytes, oligodendrocytes, or microglia.

Motoneuronal expression of neurotrophin receptor mRNA following peripheral nerve injury is summarized in Figure 1-2B. Motoneurons do not express nor upregulate trkA after injury (Escandon et al., 1994). *In situ* hybridization analysis of full length trkB mRNA shows that it does not begin to increase until 2 days after facial nerve axotomy, peaks at a 3 fold increase by seven days, and remains elevated between 1.5 and 3 fold 14 and 21 days after injury (Kobayashi et al., 1996). Expression of trkB mRNA following sciatic nerve axotomy follows a similar time course (Hammarberg et al., 2000). In contrast, although trkC mRNA remains relatively unchanged following sciatic nerve axotomy, it is massively downregulated after sciatic nerve avulsion, and remains well below intact levels at least 42 days after injury (Hammarberg et al., 2000). Thus it is possible that only more severe injuries cause downregulation of trkC. This is consistent with RT-PCR analysis of inserted and non-inserted isoforms of trkC which suggest that seven days after facial nerve axotomy (which is considered more severe than a sciatic axotomy due to its proximity to the cell body), all isoforms are downregulated below contralateral intact control levels (Fernandes et al., 1998).

Expression of the p75 receptor is barely detectable in adult motoneurons, but is rapidly upregulated following injury, with mRNA levels reaching 12 fold greater levels 7 days after axotomy (Raivich & Kreutzberg, 1987; Yan & Johnson, 1988; Ernfors et al., 1989; Koliatsos et al., 1991; Rende et al., 1992, 1995). This is supported by upregulation of p75 immunoreactivity in adult sciatic motoneurons 7 days after axotomy (Friedman et al., 1995; Rende et al., 1995). The increased expression of p75 is maintained for several weeks after axotomy, returning to baseline by 30 days (Rende et al., 1995). Interestingly, a crush injury induced a larger and more sustained increase in p75 expression than transection injury (Rende et al., 1995)

In summary, based on the expression of neurotrophins and their receptors (Figure 1-2A,B), a strong role is suggested for BDNF in mediating motoneuronal response to injury. As BDNF, trkB, and p75 are rapidly upregulated in axotomized motoneurons, BDNF may play an autocrine or paracrine role in motoneuronal response to axotomy, but the exact function of BDNF and the receptors which mediate these effects remain to be determined.

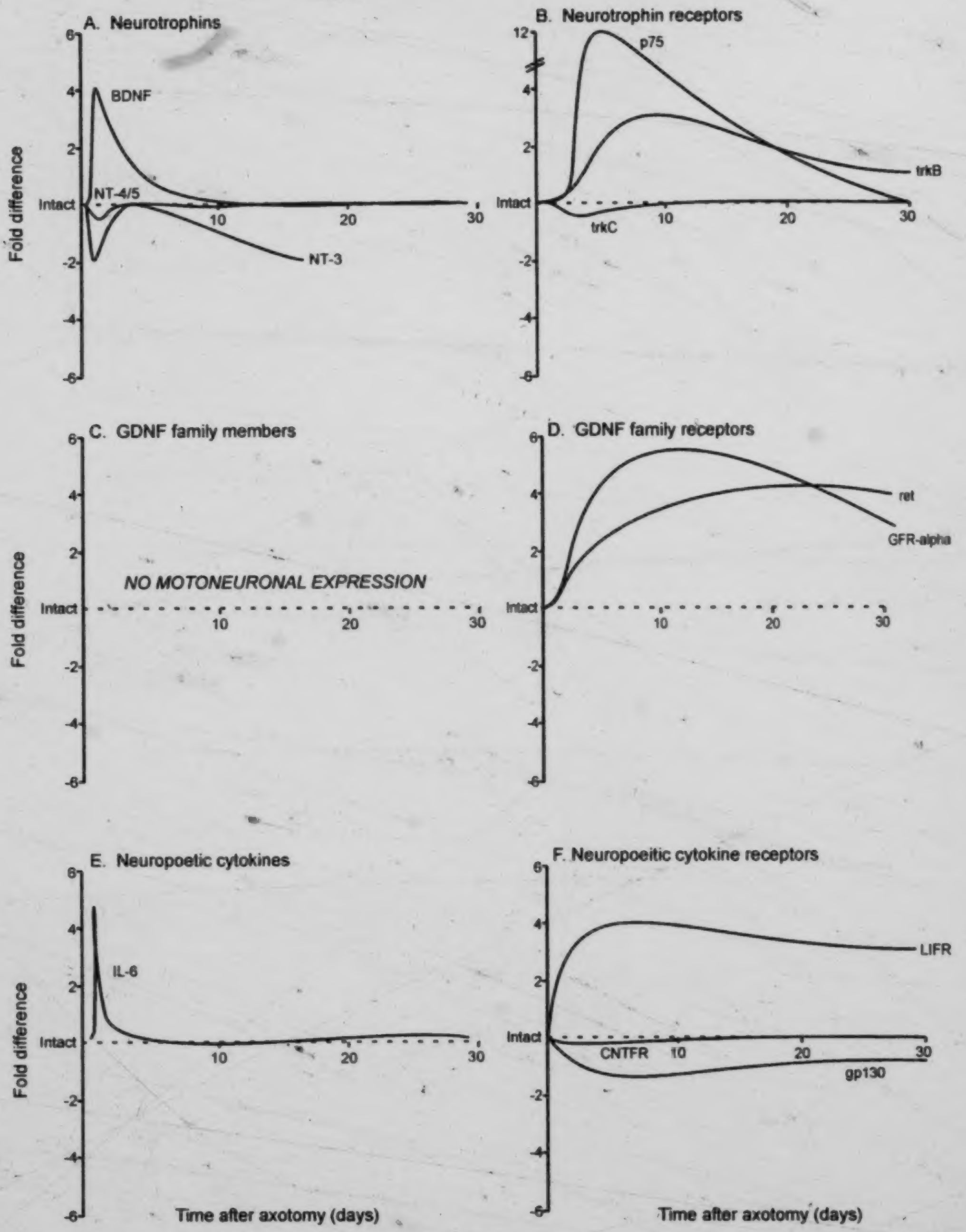
1.4.2.2: GDNF and its receptors

Although the regulation of GDNF and its receptors in response to motoneuronal injury has been well documented (Trupp et al., 1997; Burazin & Gundlach, 1998; Tsujino et al., 1999; Hammarburg et al., 2000), the role of other GDNF family members, such as NTN, ART, and PSP remains to be determined. In contrast to members of the neurotrophin family, such as BDNF, GDNF mRNA has not been detected in intact or injured motoneurons (Figure 1-2C; Naveillhan et al., 1997; Trupp et al., 1997; Burazin et al., 1998). Thus, it is not likely that GDNF mediates its effects on motoneurons in an autocrine fashion.

Motoneuronal responsiveness to GDNF is conferred by GFR-α1 and Ret mRNA upregulation as early as 18 hours after hypoglossal nerve transection, expression which peaks at 5 days, and remains elevated for at least 5 weeks post injury (Tsuijino et al., 1999). The kinetics are slightly longer in duration following facial nerve axotomy (Tsujino et al., 1999), and longer still after sciatic nerve axotomy (Figure 1-2D; Hammarberg et al., 2000), with 1) ret mRNA increasing ~2 fold, 3 days following sciatic nerve transection, peaking at ~4.5 fold at 21 days, and returning near baseline 42 days later, and 2) GFR-α1 mRNA increasing 2 fold by 1 day, peaking at ~6 fold between 7 and 14 days, and returning to baseline 42 days later (Hammarberg et al., 2000). It is not clear whether this discrepancy can be attributed to

Figure 1-2: Temporal expression of neurotrophic factors and their receptors in axotomized motoneurons. All values are normalized to contralateral intact control motoneurons (see text for details)

Figure 1-2: Motoneuronal expression of neurotrophic factor and Receptor mRNA after peripheral nerve injury



differences in various populations of motoneurons or species differences.

1.4.2.3: Neuropoeitic cytokines and their receptors

In comparison to the well characterized temporal kinetics of motoneuronal expression of neurotrophin family members, less is known about the regulation of neuropoeitic cytokines after injury. It is known that IL-6 mRNA is rapidly upregulated after facial nerve axotomy with a peak at 24 hours, and a return to baseline shortly thereafter (Figure 1-2E; Kiefer et al., 1993). Motoneuronal regulation of neuropoeitic cytokine receptors after axotomy appears to depend greatly on both the nature as well as the location of the injury. After axotomy of the facial nerve in neonatal animals, motoneuronal CNTFR mRNA was not detected (Duberly & Johnson, 1996), consistent with very little change in sciatic motoneuronal expression of CNTFR following axotomy (Hammarberg et al., 2000). In contrast, CNTFR is downregulated after adult facial nerve axotomy (Haas et al., 1999). gp130, the common signal transduction subunit for the neuropoetic cytokines is unchanged after adult facial and hypoglossal nerve transection (Haas et al., 1999; Schwaiger et al., 2000), but drastically downregulated 3 days after sciatic nerve transection, and virtually abolished after sciatic ventral root avulsion (Hammarburg et al 2000). In contrast, LIFR is upregulated 1 and 3 days after adult facial and sciatic axotomy, respectively, and remains elevated for at least 6 weeks (Haas et al., 1999; Hammarburg et al., 2000).

1.4.2.4: Intracellular signal transduction molecules

As described above in Section 1-2, there are several key intracellular proteins molecules which serve as common signal transduction molecules for the neurotrophin family, GDNF family members, and neuropoetic cytokines. These molecules include elements of the ras-erk pathway, the PI3K pathway, as well as the JAK-STAT pathway activated by the neuropoetic cytokines (Figure 1-1). In addition to the regulation of neurotrophic factors and their receptors, axotomized motoneurons also show a characteristic regulation of downstream signal transduction molecules. Specifically, shc (Tanabe et al., 1998), ras (Kiryu et al., 1996), PI3K (Ito et al., 1996), and Akt (Owada et al., 1997) all demonstrate transient mRNA upregulation following hypoglossal nerve injury. This provides strong evidence for the involvement of the PI3K-Akt pathway, and to a lesser extent the ras-erk pathway, in mediating the motoneuronal response to injury. The role of the ras-erk pathway is weakened by distinct downregulation of Grb2 (Kiryu et al., 1996), the adaptor protein involved in linking activated trkB to the ras-erk pathway (Atwal et al., 2000). In addition, facial and hypoglossal axotomy also induce upregulation of JAK2 and JAK3 mRNA upregulation, as well as phosphorylation and nuclear translocation of STAT3 (Haas et al., 1999; Schwaiger et al., 2000), indicative of neuropoetic cytokine signal transduction via gp130 or LIFR (reviewed in Ihle, 2001).

1.4.3: Distal nerve stump

1.4.3.1: Introduction

The cellular and molecular changes which occur in the nerve stump distal to the site of injury are initially degenerative, with phagocytosis of myelin and axonal debris initially by Schwann cells, then by invading macrophages; a process that is collectively known as Wallerian degeneration (reviewed in Fu & Gordon, 1997). After injury, Schwann cells of the distal nerve stump rapidly convert from a mitotically quiescent myelinating phenotype to a rapidly proliferating non-myelinating phenotype and upregulate many growth associated proteins, including neurotrophic factors, cell adhesion molecules, and many basement membrane components (Fu & Gordon, 1997). Proliferating Schwann cells form linear bands within the endoneurial sheath, known as Bands of Bungner, which are important in guiding regenerating axons across the injury site and into the distal nerve stump (reviewed in Bunge et al., 1989; Fu & Gordon, 1997). The temporal expression of 1) neurotrophins, 2) GDNF-family neurotrophic factors, and 3) neuropoetic cytokines, as well as their receptors in the distal nerve stump following injury is summarized in Figure 1-3 and will be described in detail in the following sections. It is important to note the change in the scale for the change in expression is substantially larger for Figure 1-3, compared to Figure 1-2, suggesting that the changes in neurotrophic factor and receptor expression is much more dynamic in the distal nerve stump than in the axotomized motoneurons. A summary of the axotomy-induced changes in motoneuron, and the distal nerve stump can be found in Table 1.

1.4.2.2: Neurotrophins and their receptors

The temporal expression of NGF and BDNF in the rat sciatic nerve following injury have been well characterized (Heumann et al., 1987a,b; Meyer et al., 1992). Using Northern blot analysis, NGF mRNA is barely detectable in intact nerve, but shows a biphasic upregulation after sciatic nerve transection; being rapidly upregulated 10 fold compared to intact nerve as early as 12 hours after injury, falls at two days, and returns to at least 5 fold elevated levels at 3 days and persists for at least three weeks thereafter (Figure 1-3A; Meyer et al.,1992). The second phase of NGF mRNA elevation is likely induced by IL-1β secreted by macrophages, as 1) this second phase is temporally correlated with macrophage invasion (Heumann et al., 1987), 2) can be mimicked *in vitro* by addition of activated macrophages or recombinant IL-1β (Ibid), and 3) can be blocked by IL-1β antibodies (Heumann et al., 1987; Lindholm et al., 1987).

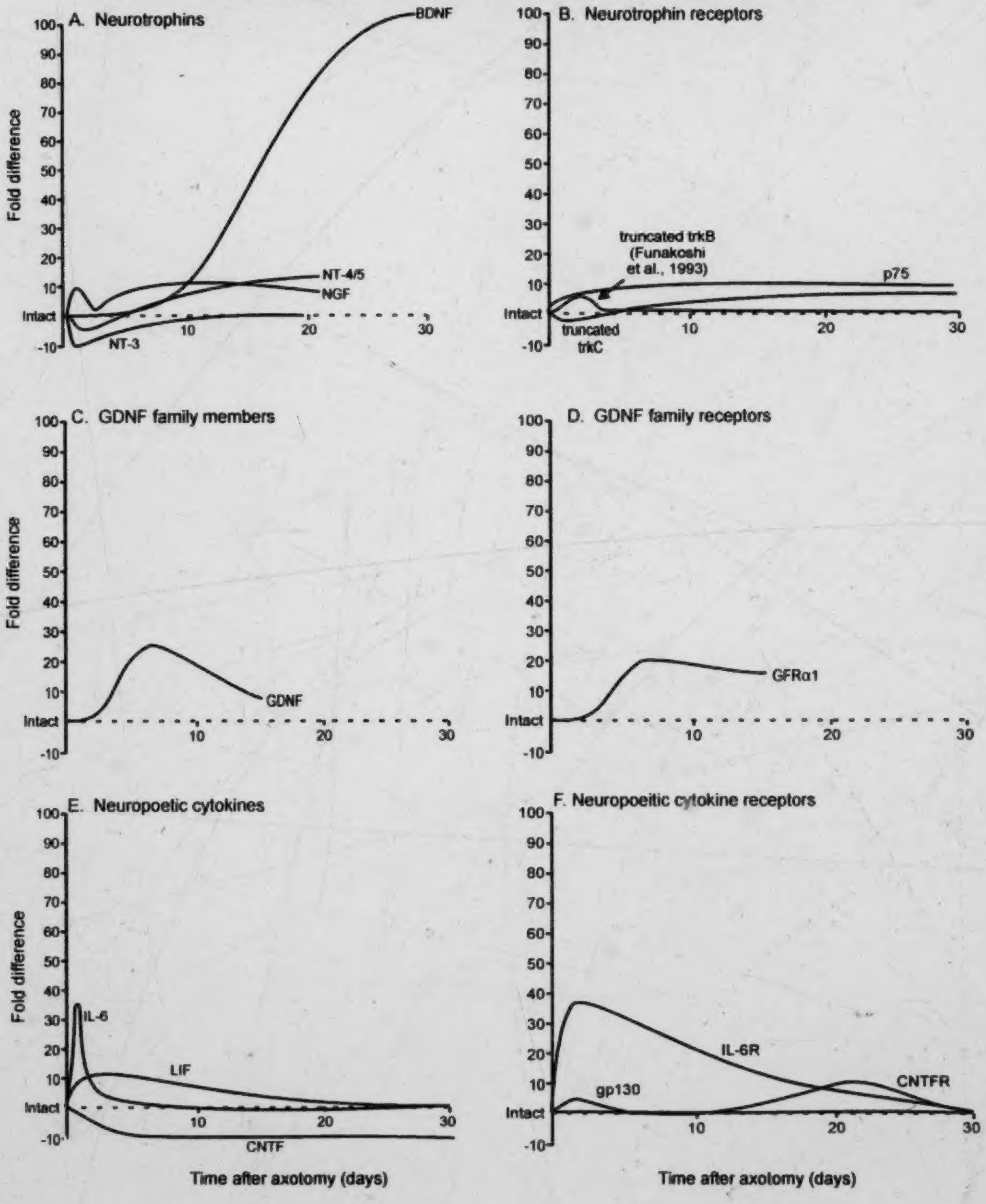
The kinetics of BDNF mRNA upregulation in the distal nerve stump following injury are substantially slower than what has been observed for NGF (Meyer et al., 1992). Like NGF mRNA, BDNF mRNA is expressed at very low levels in intact nerve (Funakoshi et al., 1993). An upregulation of BDNF mRNA is detectable at 7 days after sciatic nerve transection, and continues to increase up to 28 days later (Figure 1-3A; Meyer et al., 1992; Funakoshi et al., 1993). Furthermore, the maximal levels of BDNF mRNA are at least 10 times higher than what was observed for NGF mRNA (Meyer et al., 1992).

In comparison to what is known about temporal expression of NGF and BDNF mRNA in the distal nerve stump after injury, less is known about other members of the neurotrophin family, namely NT-3 and NT-4/5. Using RNAse protection assay, NT-3 mRNA is clearly detectable in intact nerve, but is rapidly downregulated 9 fold 6 and 12 hours after sciatic nerve transection, and progressively returns to baseline by 2 weeks later (Figure 1-3A; Funakoshi et al., 1993). In the same study, NT-4/5 mRNA showed a similar, albeit smaller, decrease in expression 6 and 12 hours after injury, but increased progressively thereafter with eightfold higher levels than in control nerves by 2 weeks post-injury.

Although the non-neuronal cells of the distal nerve stump do not express full length trkA, trkB, or trkC receptors, they do express truncated trkB and trkC isoforms, as well as

Figure 1-3: Temporal expression of neurotrophic factors and their receptors in the non-neuronal cells of the distal nerve stump. All values are normalized to contralateral intact control nerves.

Figure 1-3: Expression of neurotrophic factor and receptor mRNA in the distal nerve stump after peripheral nerve injury



the p75 receptor after injury (Figure 1-3B; Barbacid, 1994; Fu & Gordon, 1997). Truncated trkB receptor mRNA increases slightly in the distal nerve stump 1 day after sciatic nerve transection and returns to baseline by 3 days (Figure 1-3 B; Funakoshi et al., 1993). Another study demonstrated that truncated trkB expression decreases after peripheral nerve injury (Frisen et al., 1993). The reasons for this discrepancy are unclear, but the changes in truncated trkB receptor mRNA were relatively mild in both cases. Truncated trkC receptor mRNA decreases slightly in the distal nerve stump 6 and 12 hours after sciatic nerve transection, recovers to baseline, and continues to increase to 2 fold elevated levels by 2 weeks after injury (Funakoshi et al., 1993).

The p75 receptor is not expressed by mature, myelinating Schwann cells, but is rapidly upregulated in the distal nerve stump as early as 36 hours after peripheral nerve transection (Heumann et al., 1987ab; Taniuchi et al., 1988; Toma et al., 1992; Robertson et al., 1995). At one week after injury, p75 mRNA expression is increased approximately 9-fold, and continues to increase at one month to a peak of 12 fold compared to background levels (Figure 1-3B; You et al., 1996). The p75 mRNA levels are maintained by denervated Schwann cells for at least 4 months, at which time they return to basal levels (Ibid).

It was initially thought that non-neuronal expression of neurotrophin receptors, like p75 and the truncated trkB and trkC receptors may function to "present" regenerating axons with neurotrophins (Barbacid , 1994; Taniuchi et al., 1986, 1988). However, these neurotrophin receptors on the non-neuronal cells of the distal nerve stump may play a much more complex role in peripheral nerve regeneration. For example, expression of p75 on the Schwann cells has been linked to cellular migration (Anton et al., 1994) and apoptosis (Ferri & Bisby, 1999). The apoptotic signal via p75 in Schwann cells is independent of the anti-apoptotic protein Bcl-2 (Soilu-Hanninen et al., 1999). In terms of the truncated trk receptors, their role may not be presenting regenerating axons with neurotrophins, in fact, the opposite may be true. A substrate of fibroblasts stably expressing truncated trkB receptors actually inhibits neurite outgrowth by endocytosing bound neurotrophin and essentially removing trkB ligands from the environment of a growing neurite (Fryer et al., 1997). Thus expression of truncated trk receptors on the non-neuronal cells of the distal nerve stump may restrict axonal growth and regeneration.

1.4.2.3: GDNF family members and their receptors

Similar to motoneuronal response to injury, the expression of GDNF and its receptors has been well characterized in the distal nerve stump following injury, but the pattern of expression for other GDNF family members remains to be determined (Figure 1-3C,D). Using an RNAse protection assay, GDNF mRNA was detected in intact nerve, and its levels were found to increase in the distal nerve stump, peak at 7 days, and remain elevated for at least 2 weeks following sciatic nerve crush in adult mice (Naveillhan et al., 1997). It is not known however, whether or not GDNF mRNA expression would follow a similar time course following sciatic nerve transection.

Sciatic nerve crush (Naveillhan et al., 1997), and transection (Trupp et al., 1997) induces a marked increase in GFRα1 mRNA n the distal nerve stump 3 days post injury, peaking at 7 days (21 fold increase above intact), and remained elevated by 12 fold at least 14 days after injury, whereas ret mRNA was not detectable in either intact or injured nerves (Figure 1-3D; Naveillhan et al., 1997; Trupp et al., 1997). Two models have been proposed to explain this discordant expression of GFRα1 and ret in the distal nerve stump following injury. First, it has been suggested that GFRα1 expression on Schwann cells of the distal nerve stump serves to "present" regenerating axons with GDNF molecules in a manner analogous to one of the proposed functions of the p75 receptor on Schwann cells (Naveillhan et al., 1997; Trupp et al., 1997). It has also been proposed that soluble GFRα1 is secreted by Schwann cells to bind with GDNF and form a ligand-coreceptor complex, that can activate ret receptors on regenerating axons in *trans* (Naveillhan et al., 1997). However, *trans*-activation of ret is much less effective in eliciting biological responses, such as survival, than *cis*-activation (Tansey et al., 2000).

1.4.2.4: Neuropoeitic cytokines

The temporal expression of CNTF, LIF, and IL-6, as well as their receptors, following either nerve crush or nerve transection has been recently described by Ito et al.(1998) and is summarized in Figures 1-3D,E. Briefly, using RT-PCR relatively high levels of CNTF mRNA were detected in intact nerve, but LIF and IL-6 mRNA were undetectable. After injury, CNTF mRNA declines as early as 1 day (Friedman et al., 1992; Sendtner et al., 1992; Seniuk et al., 1992), and continues to fall to a 5 fold decrease compared to intact levels by one week (Ito et al., 1998). In the crushed nerve CNTF mRNA levels recovered by 4 weeks, but remained low after transection (Ito et al., 1998). In contrast, LIF and IL-6 mRNA are upregulated in the distal nerve stump (Seniuk et al., 1992; Curtis et al,. 1994; Reichert et al., 1996; Ito et al., 1998). LIF mRNA rapidly increased 10 fold 1 day after both crush and transection injuries, and returned to baseline by 1 month later (Ito et al., 1998). IL-6 expression was more transient, increasing 35 fold compared to intact levels 1 day after injury, and returned to baseline 24 hours later (Ito et al., 1998).

The rapid decline in CNTF mRNA may be compensated for by the upregulation of CNTFRα mRNA, its ligand binding receptor, which increases gradually reaching a 10 fold increase by day 21, then returning to baseline levels by day 28 (Ito et al., 1998). IL-6α mRNA also demonstrates a massive upregulation in the distal nerve stump after injury. Peak levels were observed at 2 days, reaching 8 and 35 fold elevations compared to intact levels after nerve crush and nerve transection, respectively, and again, returning to baseline by 28 days later (Ito et al., 1998). Little change was seen in the ligand binding receptor for LIF, LIFRβ (Ito et al., 1998). gp130, the common signal transduction component for CNTF, IL-6, and LIF, showed only a transient increase in mRNA expression.

The distinct temporal expression of these neuropoeitic cytokines and their receptors in the distal nerve stump suggests the possibility that CNTF, IL-6, and LIF may have discrete roles in mediating peripheral neuronal as well as non-neuronal responses to injury.

For example, as CNTF lacks a secretory sequence (see above *Structure of neuropoetic cytokines*), it is thought that nerve injury damages Schwann cells and causes the release of CNTF into the environment of regenerating axons, thus acting as a "lesion factor" (Sendtner et al., 1992, 1997). On the other hand, the massive, but transient, increase in IL-6 expression may serve to initiate a local inflammatory response, such as recruitment of macrophages to the site of injury (reviewed in Oppenheim and Saklavata, 1993). In addition to being a potent survival factor for many populations of injured neurons (Martinou et al., 1992; Curtis et al., 1994), LIF also plays an important role in promoting local axonal sprouting after injury, as cultured DRG neurons failed to show normal axonal sprouting in response to predegenerated nerves isolated from LIF homozygous knockout mice (Ekstrom et al., 2000).

Table 1: Summary of axotomy-induced changes in motoneurons and the distal nerve stump

Location	**Peptide Phenotype**	**Neurotrophins**	**GDNF family**	**Neuropoetic cytokines**
Motoneurons	ChAT↓ AChE↓ VAMP-1↓ neurofilament ↓ CGRP ↑ VAMP-2 ↑ GAP-43 ↑ tubulin ↑ actin ↑	BDNF ↑ trkB ↑ p75 ↑ NT-3↓ NT-4/5↓ trkC↓	ret ↑ GFR-α1 ↑	IL-6 ↑ LIFR ↑ CNTFR↓ gp130↓
distal nerve stump		NGF ↑ BDNF ↑ p75 ↑ NT-3↓ NT-4/5↓ tr. trkB↓ tr. trkC↓	GDNF ↑ GFR-α1 ↑	IL-6 ↑ LIF ↑ gp130 ↑ CNTFR ↑ IL-6R ↑ CNTF↓

1.5: *IN VIVO* EFFECTS OF NEUROTROPHIC FACTORS AFTER PERIPHERAL NERVE INJURY: SURVIVAL, REVERSING THE EFFECTS OF INJURY, AXONAL SPROUTING, REGENERATION, AND FUNCTIONAL RECOVERY

1.5.1: Survival

1.5.1.1: Introduction

Neuronal survival after injury depends on several factors, such as neuron type, developmental stage, and the degree and proximity of the injury (Fu & Gordon, 1997). Generally, sensory neurons are more susceptible than motoneurons and neonatal neurons are more susceptible than adult neurons. Further, more traumatic injuries induce more cell death. Injuries close to the cell body are more damaging than distal injuries. For example, in the case of adult spinal motoneurons, following distal injury, they often remain viable for long periods of time, whereas an avulsion injury may induce substantial cell death in the same cell type (Kishino et al., 1997; Novikov et al., 1997; Novikova et al., 1997; Hammarburg et al., 2000). As described above, a mosaic of neurotrophic factors are available to injured neurons from both autocrine and paracrine sources. *In vivo* exogenous application of many of these neurotrophic factors have been demonstrated to support the survival of both sensory and motoneuronal cell populations.

Although adult lumbar motoneurons rarely die after peripheral nerve injury, and remain viable up to a year after peripheral nerve injury (Carlsson et al., 1979; Gordon et al., 1991; Vanden Noven et al., 1993), substantial motoneuronal cell death follows: 1) avulsion of adult lumbar motoneurons 2) axotomy of neonatal lumbar motoneurons, and 3) and axotomy of both adult and neonatal cranial motoneurons, death being more severe in the latter (see Fu & Gordon, 1997 and references cited therein). Neurotrophic factors have been demonstrated to promote the survival of axotomized or avulsed motoneurons in many different experimental paradigms. The methods of administering neurotrophic factors range from exogenous application, adenoviral transfer, lentiviral transfer, as well as several other strategies to ensure sustained continuous release to produce long-lasting survival.

1.5.1.2: Neurotrophins:

Application of NGF to axotomized neonatal rat sciatic (Miyata et al., 1986), and facial (Sendtner et al., 1992) nerves may actually potentiate motoneuronal cell death, an effect attributed to NGF binding to p75 receptors, as motoneurons do not express trkA (Henderson et al., 1993). This effect however, may be species specific, as NGF did not potentiate motoneuronal cell death following facial nerve lesion in several strains of neonatal or adult mice; only in mice which had a conditioning crush lesion to the sciatic nerve did application of NGF induce motoneuronal cell death (Terrado et al., 2000).

It has been well documented that BDNF acts as a survival factor for injured mammalian motoneurons (Sendtner, 1996). Exogenous BDNF rescues 1) axotomized neonatal facial (Sendtner et al., 1992; Koliatsos et al., 1993; Yan et al., 1993), 2) axotomized neonatal sciatic (Yan et al., 1993; Vejsada et al., 1995; 1998), and 3) adult lumbar motoneurons following avulsion injury (Kishino et al., 1997; Novikova et al., 1997; Novikov et al., 1997). The ability of BDNF to promote survival of axotomized neonatal motoneurons likely depends on functional trkB receptors, as axotomy-induced motoneuronal cell death is significantly exacerbated in neonatal mice carrying a homozygous deletion in full length trkB receptors (Alcantara et al., 1997). The survival promoting effects of exogenous BDNF on axotomized neonatal sciatic motoneurons has been demonstrated to be transient. For example, four to five weeks after lesion, up to 95% of the motoneurons which were originally rescued by exogenous BDNF had died, despite continuous treatment (Vejsada et al., 1998). In contrast, continuous exogenous BDNF exerted long term survival promoting effects on adult motoneurons after sciatic nerve avulsion (Kishino et al., 1997). As with isolated embryonic motoneurons (Wiese et al., 1999), the effects of BDNF on motoneuronal survival after sciatic nerve axotomy are dose-dependent, with rescue effects of high doses being significantly lower compared to optimal doses (Vejsada et al., 1995; 1998).

There is considerable controversy concerning the relative potency of NT-3 and NT-4/5 in promoting the survival of injured motoneurons *in vivo* (Sendtner et al., 1996). In one study, NT-3 AND NT-4/5 were as effective as BDNF in promoting the survival of axotomized neonatal sciatic motoneurons (Vejsada et al., 1995). In other studies, NT-3

(Hughes et al., 1993; Yan et al., 1993) and NT-4/5 (Hughes et al., 1993) have been demonstrated to promote the survival of axotomized neonatal cranial and lumbar motoneurons, but to a lesser extent than BDNF. In contrast, Koliatsos et al. (1993) reported that NT-3 did not promote the survival of axotomzied neonatal facial motoneurons compared to controls. These discrepancies are difficult to reconcile, but may be due to slight variations in experimental procedures, or methods of evaluating survival of axotomized motoneurons.

1.5.1.3: GDNF family members

There is considerable evidence that GDNF promotes the survival of several motoneuronal populations after injury. In neonatal animals, exogenous GDNF promotes the survival of axotomized facial (Henderson et al., 1995; Yan et al., 1995), and sciatic (Oppenheim et al., 1995; Vejsada et al., 1998; Yuan et al., 2000) motoneurons, in addition to sciatic motoneurons following ventral root avulsion (Yuan et al., 2000). In adult animals, exogenous GDNF rescues axotomized rat facial motoneurons (Yan et al., 1995) in addition to avulsed sciatic motoneurons (Li et al., 1995). However, like BDNF, a single local dose of exogenous GDNF only produced a transient survival promoting effect (Vejsada et al., 1998). Thus to promote long term survival, methods to obtain a more sustained release of GDNF have been examined, such as polymer-encapsulated cells which secrete GDNF (Vejsada et al., 1998), adenoviral- (Baumgartner & Shine, 1998ab; Sakamoto et al., 2000; Watabe et al., 2000) and lentiviral-mediated gene transfection (Hottinger et al., 2000) of motoneurons with GDNF. Adenviral transfection of adult facial (Sakamoto et al., 2000) and cervical (Watabe et al., 2000) motoneurons protected these motoneurons from axotomy- and avulsion induced cell death. Lentiviral mediated transfection of adult facial motoneurons conferred nearly complete (~95%) survival effects which lasted as long as 3 months (Hottinger et al., 2000).

Although there is sufficient evidence that NTN and PSP support motoneuronal survival *in vitro* (Milbrandt et al., 1998; Cacalano et al., 1998, Enomoto et al., 1998) it remains to be determined whether or not other members of the GDNF family also prevent axotomy-induced cell death.

1.5.1.4: Neuropoeitic cytokines

Although exogenous application of IL-6 is ineffective in promoting the survival of axotomized neonatal motoneurons (Li et al., 1994), there is extensive evidence that CNTF and LIF are potent survival factors for axotomized neonatal motoneurons *in vivo* (Sendtner et al., 1990, 1992, 1997; Clatterbuck et al., 1993; Hughes et al., 1993; Cheema et al., 1994; Li et al., 1994). Similar to members of neurotrophic factor families, such as BDNF and GDNF, the effects of exogenous CNTF on motoneuronal survival are transient (Vejsada et al., 1995). Thus strategies other than exogenous administration have been employed to provide long-term continuous administration of CNTF. Adenoviral transfer of CNTF elicited long-term protection of axotomized facial motoneurons, and when combined with adenoviral transfer of BDNF, showed significantly better long-term protection (Gravel et al., 1997)

1.5.2: Reversing the effects of injury

In response to nerve injury, there are many changes which occur in motoneurons, which has been denoted as a transition from a mature "transmitting" to a "regenerating" phenotype (Fu & Gordon, 1997). In particular, motoneurons downregulate enzymes associated with neurotransmission, for example: ChAT (Borke et al., 1993; Rende et al., 1995; Yan et al., 1995; Friedman et al., 1995; Tuszynski et al., 1996; Kishino et al., 1997; Jacobsson et al., 1998), and acetylcholinesterase (AChE; Fernandes et al., 1998; Kishino et al., 1997), as well as proteins associated with transmitter release, such as the synaptobrevin isoforms vesicle-associated membrane protein (VAMP)-1 (Jacobsson et al., 1998). In close temporal correlation with the decline in ChAT and AChE is an upregulation of regeneration associated genes, such as actin, GAP-43, and Tα1-tubulin (Miller et al., 1989; Tetzlaff et al., 1991; reviewed in Bisby & Tetzlaff, 1992), in addition to calcitonin gene related peptide (CGRP; Borke et al., 1993; Rende et al., 1995; Jacosson et al., 1998), as well as neurotrophic factors and their receptors (see Figure 1-2, and above: *Spatio-temporal expression of neurotrophic factors after peripheral nerve injury-Motoneurons*).

In addition to the phenotypic changes in motoneurons after injury, there are a number of electrophysiological changes which occur, such as decline in axonal conduction velocity, rheobase, EPSP amplitude, duration of afterhyperpolarization, and increase in input resistance (Mendell et al., 1995; Munson et al., 1997)

There is substantial evidence that BDNF and NT-4/5, but not NGF or NT-3, play important roles in maintaining the cholinergic phenotype after injury. For instance, intrathecal administration of BDNF prevents the loss of ChAT and AChE in motoneurons after sciatic nerve avulsion in a dose-dependent fashion (Kishino et al., 1997, 1998). Intracerebralventricular infusion of BDNF and NT-4/5, but not NT-3 or NGF maintained the expression of ChAT in axotomized hypoglossal motoneurons (Tuszynski et al., 1996). Also, peripheral application of BDNF and NT-4/5 maintained the expression of ChAT in axotomized sciatic motoneurons (Friedman et al., 1995). The ability of BDNF and NT-4/5, but not NGF or NT-3, to promote the cholinergic phenotype after injury is consistent with motoneuronal regulation of neurotrophin receptors after injury, which rapidly upregulate trkB, but not trkC or trkA (see section 1.4.2.1). It is not clear however, if and how this regulation of ChAT/AChE expression is linked to the other biological effects of BDNF and NT-4/5 *in vivo*, such as sprouting and axonal regeneration (see below).

In contrast to the regulation of the cholinergic phenotype which seems to depend on only trkB ligands, maintaining axonal conduction velocity in axotomized motoneurons depends on both trkB and trkC ligands, despite the downregulation of NT-3 and trkC by axotomized motoneurons (see section 1.4.2.1) as peripheral application of NT-4/5 or NT-3 improved motor axonal conduction velocity 5 weeks after tibial nerve axotomy (Munson et al., 1997). Furthermore, reducing levels of endogenous trkB and trkC ligands using IgG sequestering molecules reduced motor axonal conduction velocity to a level similar to what is observed after axotomy (Ibid). In contrast to the neurotrophins, little is known about the roles of GDNF family members and neuropoetic cytokines in reversing the effects of motoneuronal injury, such as regulation of cholinergic enzymes and conduction velocity. However, in light of the considerable overlap in the signal transduction mechanisms used by these three families of neurotrophic factors, in addition to their ability to promote the survival of injured motoneurons, one may predict a similar overlap in their ability to reverse the effects of axotomy. Surprisingly however, exogenous CNTF did not attenuate the increase in CGRP, nor strongly reverse the ultrastructural changes in motoneurons (fragmentation of rough endoplasmic reticulum, decrease in Golgi apparatus size) following facial nerve axotomy (Demitriou et al., 1996; Ulenkate et al., 1996).

1.5.3: Axonal sprouting, regeneration, and functional recovery

1.5.3.1: Introduction

Despite the compelling experiments which demonstrate that neurotrophic factors effectively promote sensory and motoneuronal survival after injury, the evidence showing a link between exogenous neurotrophic factors and axonal regeneration after injury has been largely indirect. Evaluations of peripheral nerve regeneration have relied heavily on outcomes such as axon counts distal to the site of injury, as well as functional evaluations (e.g. the "pinch test" to evaluate sensory regeneration and walking track analysis to evaluate motor regeneration). Although these approaches have the potential to be clinically relevant, they are confounded by the fact that they do not take into account axonal sprouting, and thus do not provide a direct measure of the absolute number of peripheral neurons which regenerate their axons in response to exogenous neurotrophic factors. Techniques such as counting the number of neurons which have regenerated axons through the injury site and into distal nerve stump by retrograde labelling of regenerated axons distal to the site of injury and repair have provided important insight into the mechanisms by which short periods of continuous electrical stimulation can promote the speed and accuracy of motor axonal regeneration (Al-Majed et al., 2000a). Thus, only combined with quantitative measures of axonal regeneration, such as fluorescent retrograde labelling, can these more qualitative measures of axonal regeneration (pinch test, walking track analysis, axon counts) provide a more comprehensive picture as to the functional roles of neurotrophic factors in peripheral nerve regeneration.

1.5.3.2: Neurotrophins

In line with the ability of BDNF to promote neurite outgrowth *in vitro* (Dolcet et al., 1999; Atwal et al., 2000), it has been shown that BDNF can induce motor axonal outgrowth after ventral root avulsion (Novikova et al., 1997; Novikov et al., 1997; Kishino et al., 1997), however, this regeneration was primarily restricted to the avulsion site. After less traumatic injuries, such as sciatic nerve transection and repair, BDNF did not enhance sciatic functional recovery as evaluated by sciatic function index (Moir et al., 2000), gait analysis (Shirley et al., 1996), or force recovery (Shirley et al., 1996). However, the indirect methods of evaluating functional recovery make these results difficult to interpret. For example, Fu & Gordon (1995) demonstrated that despite a 66% reduction in the number of MUs innervating the tibialis anterior muscle after long term periods of chronic axotomy, there were no observable changes in whole muscle twitch or tetanic forces. These data highlight the extensive axonal branching ability of axotomized motoneurons which functionally compensate for the reduction in motor axons which reinnervate denervated muscle. These data also demonstrate the difficulty in interpreting functional data, such as walking track analysis or force measurements alone.

The ineffectiveness of BDNF in promoting axonal regeneration after injury is surprising in light of recent evidence that the accelerated femoral motor axonal regeneration induced in response to short term electrical stimulation (Al-Majed et al., 2000a) is correlated with accelerated temporal kinetics of BDNF and trkB mRNA expression (Al-Majed et al., 2000b). For example, electrical stimulation causes the peak expression of BDNF mRNA to shift from 7 days, to as early as 2 days, with maximum values being signficantly higher following electrical stimulation (Al-Majed et al., 2000b). It is possible that accelerating motoneuronal expression of BDNF and trkB mRNA, and application of exogenous BDNF to the site of injury and repair exert distinct biological effects.

Despite the incapability of exogenous BDNF to promote functional motor recovery after peripheral nerve injury (Shirley et al., 1996; Moir et al., 2000), it is clear that endogenous BDNF is important for peripheral nerve regeneration, as application of an anti-BDNF antibody significantly reduces 1) the length of regenerating axons, 2) the number and density of myelinated axons, and 3) the amount of sensory axon regeneration, as evaluated by the "pinch test" (Zhang et al., 2000).

In contrast to the limited effectiveness of exogenous BDNF in promoting axonal regeneration after sciatic nerve repair, NT-3 delivered the site of axonal regeneration via fibronectin mats which bridged a 10 mm gap between proximal and distal nerve stumps significantly increased the number of axons which crossed the gap and penetrated the distal nerve stump compared to fibronectin mats alone (Sterne et al., 1997a). In addition, there were more myelinated axons in the NT-3/fibronectin groups 8 months after nerve repair compared with fibronectin mats alone (Sterne et al., 1997a). Using the same surgical model, it was found that the NT-3 enhanced peripheral nerve regeneration was associated with increased muscle recovery from denervation atrophy, and NT-3 acted to selectively improve the cross-sectional area of fast-type muscle fibres which express the type 2b myosin heavy chain (Sterne et al., 1997b). The ability of NT-3 to promote selective reinnervation of fast muscle fibres is supported by the fact that NT-3 increased the number and size of reinnervated neuromuscular junctions in the fast extensor digitorum longus muscle, but had no effect on reinnervation of the slow soleus muscle after sciatic nerve repair (Simon et al., 2000).

Perhaps the most compelling evidence supporting a role for neurotrophins in peripheral nerve regeneration comes from experiments using a multifunctional chimeric neurotrophin, pan-neurotrophin-1 (PNT-1), which was engineered by combining the active domains of NGF, BDNF, and NT-3 into an NT-3 backbone (Ibanez et al., 1993), and its expression after peripheral nerve injury was driven by coupling the PNT-1 gene to the fourth promoter of the BDNF gene (Funakoshi et al., 1998). Therefore, the expression of PNT-1 after injury would follow a similar time course after injury as BDNF (Figure 1-2A,1-3A; Meyer et al., 1992; Funakoshi et al., 1993; Kobayashi et al., 1996). Expression of PNT-1 after a combined sciatic crush/freeze injury initially caused increased axonal sprouting, as well as accelerated elimination of axon sprouts at later time points, suggestive of accelerated maturation of regenerated axons (Funakoshi et al., 1998). In the same study, compared to wild-type control mice, in PNT-1 transgenic mice, there was 1) increased motor and sensory innervation of the plantaris muscle and skin, respectively, 2) increased compound action potential recovery in both dorsal and ventral spinal roots, and 3) attenuated reduction in muscle mass, suggestive of increased functional motor recovery.

Despite the extensive *in vitro* evidence that neurotrophins can initiate distinct signalling cascades by binding to either trk or p75 receptors (see above), little is known about the receptors which mediate the effects of neurotrophins *in vivo*. However, evidence of increased rate of recovery of whisker movement and more axon profiles in p75 (-/-) mice compared to wild-type controls after facial nerve crush suggests the possibility that p75 may serve to restrict motor axonal regeneration (Ferri et al., 1998). In addition, a recent study by Namikawa et al. (2000) have provided new insight into the relative contributions of the ras-erk and PI3K pathways in motoneuronal survival and axonal regeneration after injury. Specifically, axotomized neonatal hypoglossal neurons transfected with a constitutively active Akt-carrying adenovirus survived better than non-transfected controls, and axotomized adult hypoglossal motoneurons showed accelerated axonal regeneration, as evaluated by an increase in the number of motoneurons retrogradely labelled via the tongue (Namikawa et al., 2000). In contrast, axotomized neonatal motoneurons expressing constitutively active mek did not show an increase in survival compared to wild-type controls (Ibid). These data are consistent with the *in vitro* evidence demonstrating a greater importance of the PI3K pathway in motoneuronal survival compared to the ras-erk pathway (Dolcet et al., 1999; *see* section1.3.1.1), and upregulation of elements of the PI3K pathway by axotomized motoneurons (*see* section 1.4.2.4).

1.5.3.3: GDNF family members

Overexpression of GDNF in developing muscle by coupling the GDNF gene to the muscle specific promoter myogenin causes hyperinnervation of motor endplates during development (Nguyen et al., 1998). This hyperinnervation is associated with motor axonal sprouting and enlargement of motor units, and not increased motoneuronal survival. Thus it is suggested that GDNF may facilitate synapse formation, or act as a “synaptotrophin”, for developing neuromuscular junctions (Ibid). This effect is specific for GDNF, as overexpression of NT-3 or NT-4/5 did not cause hyperinnervation. Adenoviral gene transefection of axotomized neonatal motoneurons with GDNF increases the numbers of myelinated axons in the facial nerve after a crush injury, and this increased number of myelinated axons is associated with significantly improved whisker function compared to non transfected controls (Baumgartner & Shine, 1998 a,b). A role for other GDNF family members in mediating axonal regeneration after injury remains to be determined.

1.5.3.4: Neuropoeitic cytokines

In line with the potent survival effects of CNTF on axotomized motoneurons (see above), there are several lines of evidence which suggest that CNTF and other neuropoetic cytokines, either alone, or in combination with other neurotrophic factors, play important roles in mediating motor axonal sprouting and functional recovery after peripheral nerve injury.

Based on the ability of exogenous CNTF to induce motor axonal sprouting CNTF at motor endplates (Gurney et al., 1992), Siegel et al. (2000) investigated the role of CNTF in axonal sprouting following partial denervation. Partial denervation normally induces a situation in which intact motor axons sprout axon collaterals to innervate denervated motor endplates (Edds, 1950). Sprouting does not occur in CNTF homozygous knockout mice following partial denervation, however, this sprouting can occur if exogneous CNTF is applied to the denervated muscle of CNTF knockout mice (Siegel et al., 2000).

Exogenous CNTF and BDNF (Lewin et al., 1997), or CNTF and BDNF covalently linked to collagen tubules (Ho et al., 1998), have been shown to slightly improve sciatic functional recovery after transection and repair as measured using walking track analysis. However, this modest improvement was not associated with increases in axonal conduction velocity (Ho et al., 1998) or axon diameter (Lewin et al., 1997; Ho et al., 1998).

IL-6 and IL-6R have been shown to be important in motor axonal regeneration (Hirota et al., 1996). Intraperitoneal injection of an antibody which prevents IL-6 binding to IL-6R, and thus restricts retrograde transport of IL-6 to the injured motoneurons, significantly reduces the number of hypoglossal motoneurons which regenerate their axons. In the same study, transgenic mice overexpressing IL-6, IL-6R, or both, showed accelerated motor axonal regeneration compared to non-transgenic controls.

The role of neuropoetic cytokines in peripheral nerve regeneration has also been evaluated using knockout mice for CNTF (Yao et al., 1999) and IL-6 (Zhong et al., 1999; Inserra et al., 2000). IL-6 knockout mice showed delayed regeneration after nerve crush injury compared to wild-type controls (Zhong et al., 1999; Inserra et al., 2000), but recovered to the same extent at longer periods of regeneration (Inserra et al., 2000). IL-6 knockout mice also demonstrated deficits in sensory, but not motor, compound action potentials after sciatic nerve crush (Zhong et al., 1999). In contrast to the IL-6 knockout mice which eventually demonstrated functional recovery after nerve crush injury (Inserra et al., 2000), CNTF knockout mice did not show this recovery after nerve crush even after long periods of regeneration (Yao et al., 1999). There was no difference in recovery between IL-6 knockout mice and controls, or CNTF knockout mice and controls, after nerve transection and repair (Yao et al., 1999; Inserra et al., 2000).

In summary, these studies support a role for neuropoetic cytokines, such as IL-6 and CNTF, in recovery of denervated muscles after nerve injury, but the exact nature of this role is unclear. By relying primarily on functional analysis, it is unknown whether neuropoetic cytokines are important in increasing the number of motoneurons which regenerate their axons, increasing the rate of motor axonal regeneration, or increasing either regenerative or terminal sprouting of motor axons. Further investigation is required to provide clear and well delineated mechanisms by which neuropoeitic cytokines mediate their effects on motoneurons during axonal regeneration and muscle reinnervation.

1.6: Conclusions and study objectives

Despite the clear evidence that neurotrophic factors promote motoneuronal survival after injury *in vivo*, and their well delineated intracellular pathways *in vitro*, clear quantitative evidence for their ability to promote motor axonal regeneration is lacking. The experiments described in this thesis use a quantitative method to clearly define the role of neurotrophic factors, their receptors, as well as their mechanisms of action during motor axonal regeneration and muscle reinnervation. By carefully examining the role of neurotrophic factors in the axonal regeneration of both acutely and chronically axotomized motoneurons, effective therapeutic strategies may be developed to sustain regeneration over extended periods of time to treat the poor functional recovery which follows peripheral nerve injury.

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Chapter 2: A Dose-Dependent Facilitation and Inhibition of Motor Axonal Regeneration by BDNF: The Role of p75 Receptors

2.1: Introduction

A time-dependent reduction in the capacity of axotomized motoneurons to regenerate their axons has been shown to be one of the major contributing factors to poor functional recovery after peripheral nerve injury (Fu and Gordon, 1995;1997). The processes underlying this declining regenerative capacity after chronic axotomy are not well understood, and no effective treatment strategies have been developed to sustain axonal regenerative capacity over time. Neurotrophic factors, such as the neurotrophins that are expressed after nerve injury, are important for neuronal survival and have been proposed to be involved in axonal regeneration (Meyer et al., 1992; Sendtner et al., 1992; Koliatsos et al., 1993; Kobayashi et al., 1996; Novikov et al., 1997). Neurotrophins are structurally and functionally related polypeptides that have important roles in the development, maintenance, and functional plasticity of central and peripheral neuronal populations (Thoenen, 1995; Fu and Gordon, 1997; Terenghi, 1999). BDNF is a member of the neurotrophin family, which also includes nerve growth factor (NGF), neurotrophin (NT) -3, -4/5, -6, and -7. BDNF mediates its effects by binding to two classes of receptors, the receptor tyrosine kinase trkB, and/or the p75 receptor which binds all neurotrophins with similar affinity (Barbacid, 1994).

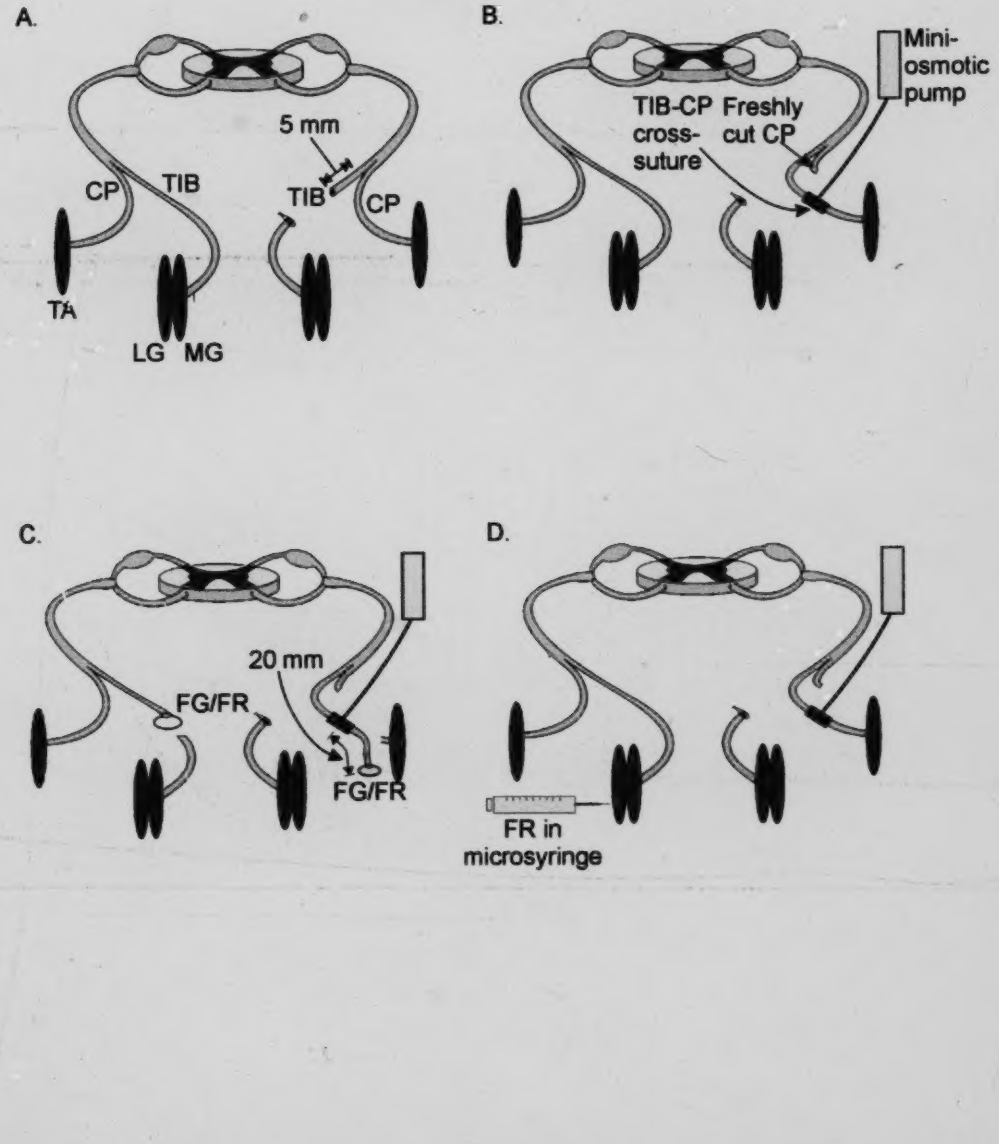
BDNF has been suggested to be a good candidate to promote motor axonal regeneration for many reasons. First, BDNF, trkB receptors, and p75 receptors are rapidly upregulated in axotomized motoneurons (Ernfors et al., 1989; Funakoshi et al., 1993; Kobayashi et al., 1996; Hammarburg et al., 2000); denervated Schwann cells also exhibit an upregulation of BDNF and p75 (Ernfors et al., 1989; Sendtner et al., 1992; Meyer et al., 1992). Second, BDNF plays an important role in the survival of axotomized neonatal motoneurons (Sendtner et al., 1992; Koliatsos et al., 1993; Vejsada et al., 1995; 1998), as well as adult motoneurons following ventral root avulsion (Novikova et al., 1997; Novikov et al., 1997; Kishino et al., 1997). Third, exogenous BDNF maintains the cholinergic phenotype in axotomized adult motoneurons by preventing downregulation of choline acetyltransferase (ChAT; Yan et al., 1992; Friedman et al., 1995; Kishino et al., 1997). Fourth, systemic application of an anti-BDNF antibody reduces the number of regenerated axons and their myelination following a sciatic nerve crush injury (Zhang et al., 2000). There have been attempts to promote peripheral nerve regeneration after sciatic nerve section and repair by exogenous application of BDNF (Lewin et al., 1996; Utley et al., 1997., Moir et al., 2000). However, the indirect assays of axonal regeneration, including the sciatic functional index and visualization of regenerated axons, showed little or no positive effects.

Currently, little information is available regarding the role that the trkB and p75 receptors play in mediating the effects of BDNF. For example, p75 receptors are rapidly upregulated in axotomized motoneurons and denervated Schwann cells (Ernfors et al., 1989; Funakoshi et al., 1993) but a physiological role for these receptors has been difficult to define. It has been suggested that the p75 expression on Schwann cells may be involved in presenting regenerating axons with neurotrophins (Taniuchi et al., 1988), Schwann cell migration (Anton et al., 1994), and Schwann cell apoptosis (Ferri & Bisby, 1999). However, recent evidence has implied that p75 may serve to inhibit axonal growth, both in sympathetic neurons (Kohn et al., 1999; Walsh et al., 1999) and motoneurons (Ferri et al., 1998).

In the present study, we use an *in vivo* model to test the hypothesis that BDNF mediates a biphasic dose-dependent effect by not only promoting motor axonal regeneration, but also inhibiting regeneration by binding to p75 receptors. Our findings demonstrate dose-dependent bimodal facilitatory and inhibitory effects of BDNF on chronically axotomized motoneurons. The latter effect was reversed by preventing BDNF binding to p75 receptors with function blocking antibodies.

Figure 2-1. Schematic representation of surgical procedures *A*. The tibial (TIB) nerve was dissected and transected 5 mm distal to the trifurcation from the common peroneal (CP) and sural (not shown) branches of the sciatic nerve. For rats in which axonal regenerative capacity was reduced by chronic axotomy, the proximal TIB stump was sutured to surrounding innervated muscle to prevent regeneration for a 2 month period. *B*. Either immediately following transection or after a 2 month period of chronic axotomy, the proximal TIB nerve stump was cross-sutured to a freshly denervated CP distal nerve stump within a 5 mm silastic nerve cuff. Twenty-eight day mini-osmotic pumps were connected to the nerve cuff via a silastic catheter and implanted under the skin of the back. *C*. Contralateral intact TIB motoneurons as well as axotomized TIB motoneurons which have regenerated their axons a distance of 20 mm are labeled with either fluorogold (FG) or fluororuby (FR). *D*. In a separate group of rats, TIB motoneurons were identified for counting and evaluating motoneuronal soma size by intramuscular injection of FR into lateral gastrocnemius (LG) and medial gastrocnemius (MG) muscles (*see text for details*).

Figure 2-1: Schematic representation of surgical procedures and drug delivery system


A.
5 mm
CP
TIB
TIB
CP
TA
LG
MG
B.
Mini-osmotic pump
TIB-CP cross-suture
Freshly cut CP
C.
20 mm
FG/FR
FG/FR
D.
FR in microsyringe

2.2: Methods

2.2.1: Animal surgery and pump implantation.

All animal procedures were conducted in accordance with the Canadian guidelines for animal experimentation, and a local animal welfare committee. Under sodium pentobarbitol anaesthesia (30 mg/kg i.p.) and, using aseptic precautions, the right sciatic nerve was exposed and the main branches were dissected in 108 adult female Sprague-Dawley rats (200-225g). The TIB nerve was transected 5 mm distal to its bifurcation from the CP and sural nerves with fine microsurgical scissors (Fig. 1A, sural not shown for simplicity). The TIB nerve stump was cross-sutured to the distal stump of a cut CP nerve either immediately (n=40) or following a 2 month period of chronic axotomy (n=68; Fig. 1B). The proximal stump of the TIB nerve was ligated and sutured to the innervated biceps femoris muscle to prevent regeneration during the period in which motoneurons remained chronically axotomized (Fu and Gordon, 1995). Nerve stumps were sutured together in a 5 mm silastic cuff (Dow Corning, 0.64 mm I.D., 1.19 mm O.D.) with a single 8-0 suture (Ethicon, Peterborough, ON) and attached to an Alzet 2ML4 (Alza Corp., Palo Alto, CA) mini-osmotic pump via a 4 cm long silastic catheter (Fig. 1B). The drug delivery catheter and 5 mm silastic nerve cuff were locally assembled such that the catheter was inserted into a small hole in the top of the nerve cuff and sealed with Reprocil Dentsply caulk (Dentsply International Inc., Milford, DE). This design was used to ensure a localized delivery of treatment solutions directly to the site of nerve injury and repair for a 28 day period, prior to evaluation of motor axonal regeneration.

The pump and catheter were anchored under the skin of the back with 4 sutures with 4-0 silk (Ethicon) to the underlying muscle. The skin was closed with 4-0 silk internal sutures. As a control for the drug delivery hardware, we performed an immediate TIB-CP nerve suture as described above in a separate group of rats (n=6) with the following exceptions: 1) nerve repair was performed using epineurial coaptation with 8-0 sutures, and 2) the drug delivery hardware (5 mm silastic nerve cuff, 4 cm silastic catheter, and Alzet mini osmotic pump) was replaced with a 5 X 20 mm piece of saline soaked gelfoam in close apposition to the repair site.

2.2.2: Delivery of exogenous BDNF and neutralizing antibodies via Alzet mini osmotic pumps.

BDNF was administered throughout the entire 28 day period of regeneration prior to retrograde labelling of the TIB motoneurons which regenerated axons into the CP nerve stump. Biological activity of BDNF at 37^0C, as determined by an *in vitro* assay of survival of embryonic Day 8 chick dorsal root ganglion neurons, declines as a function of time to 63% at 15-21 days, which plateaus thereafter (Dittrich et al., 1996) indicating that at least 63% of activity is retained during the TIB motor axonal regeneration into the freshly denervated CP nerve stump. BDNF (kindly provided by Regeneron, Tarrytown, NY) was diluted in saline (0.9%) and administered via the mini-osmotic pumps in doses between 0.1 and 20 μg/day (n=52) at a continual flow rate of 2.5 μl per hr for 28 days. In control rats, the pumps were loaded with saline alone (n =20).

To examine the role of the p75 receptors in mediating the effects of exogenous BDNF, the p75 function blocking antibody REX, prepared as an antiserum, was used together with exogenous application of BDNF (n=8). REX (a kind gift of Dr. L. Reichardt, University of California, San Francisco, CA) is directed against the extracellular domain of p75 and was used in a dilution of 1:100 to prevent BDNF binding (Weskamp and Reichardt, 1991; Kohn et al., 1999). We also administered REX alone (n=8) to evaluate any possible effects on the p75 receptor, independent of BDNF (see Weskamp and Reichardt, 1991). To control for the specificity of the REX antisera, BDNF was infused with normal rabbit serum (Sigma Chemicals, MO) as a negative control (n=4).

2.2.3: Retrograde labeling of TIB motoneurons:

Following a 28 day period of axonal regeneration and delivery of one of the above-mentioned treatment solutions, the right CP nerve was transected 20 mm distal to the TIB-CP surgical repair line for application of the fluorescent dyes to backlabel regenerated TIB motoneurons (Fig. 1C). Contralateral intact TIB motoneurons and axotomized motoneurons which regenerated their axons through the CP distal nerve sheath were labeled with either fluororuby (dextran tetramethylrhodamine; Molecular Probes, D-1817, Eugene, OR) or fluorogold (4% in cocodylic acid; Fluorochrome Inc., Denver, USA). Fluorogold and fluororuby were the 2 dyes chosen based on their ability to be effectively endocytosed and retrogradely transported (Schmued and Fallon, 1986). Backlabelling with fluorogold was done by exposing the tip of the cut CP nerve to approximately 100 µL of fluorogold for 1 hour in a vaseline well, after which it was extensively irrigated. Backlabelling with fluororuby was done by placing the tip of the cut CP nerve above a small weighing paper with fluororuby for 2 hours and then irrigating the nerve. The skin was closed using 4-0 silk (Ethicon) and the animals were allowed to recover for 6 days to allow for consistent retrograde transport of the dyes to all motoneuronal cell bodies (Sagot et al., 1998). The dyes were alternated between rats to control for possible differences in retrograde transport for each dye.

2.2.4: Animal perfusion and tissue preparation.

The rats were deeply anaesthetized with sodium pentobarbitol (60 mg/kg i.p.) for transcardial perfusion of 300 mL saline followed by 500 mL ice cold paraformaldehyde (4% paraformaldehyde in 0.1M phosphate buffer). The T11-L1 spinal segments which include the entire TIB motoneuronal pool (Swett et al., 1986) were removed. Following overnight post-fixation and cryoprotection in a 4% paraformaldehyde, 30% sucrose, the cords were frozen in liquid nitrogen and stored at -70°C.

2.2.5: Enumeration of TIB motoneurons

Longitudinal 50 µm sections were cut on a cryostat (Leica, Jung CM 3000) and mounted serially on glass slides. Each section was viewed under a Leitz-Diaplan fluorescent microscope (Fig. 2A. Fluororuby excitation band pass 515-560; B. Fluorogold excitation filter band pass 340-380 nm). Only motoneurons in which a distinct nucleus could be seen were counted. These numbers were corrected to control for the counting of split nuclei using the method of Abercrombie (1946).

2.2.6: Histological evaluation of axonal regeneration

To examine TIB axonal regeneration through the injury site and into the CP distal nerve stump, the TIB-CP nerve suture site was removed from a small subset of animals and stained with a modified Bielschowsky's method according to standard procedure. Briefly, the site of nerve injury and repair was removed and embedded in paraffin prior to microtome sectioning in 8 μm longitudinal serial sections that were mounted on glass slides. Sections were stained for 20 minutes in 20% silver nitrate ($AgNO_3$) then soaked in ammoniacal silver nitrate ($AgNO_3$ - NH_4OH) for 20 minutes. Slides were developed for approximately one minute, washed in distilled water, and toned with sodium thiosulfate for 10 minutes. The site of nerve injury and repair was viewed under light microscopy and evaluated for axonal regeneration through the injury site and into the distal CP nerve stump.

2.2.7: Evaluation of Motoneuron Survival

In a separate group of animals (n=22), TIB motoneurons were chronically axotomized for 2 months prior to suture to a freshly cut distal CP nerve stump as described above. Animal received either saline (n=8), low dose (2 μg/day; n=8) or high dose (20 μg/day; n=6). The intact TIB motoneuronal pool was identified by injecting the lateral and medial heads of the left (contralateral) gastrocnemius muscle with a 10% fluororuby solution dissolved in saline and 0.1% dimethyl sulfoxide (DMSO) 2 weeks prior to sacrifice to localize the axotomized TIB motoneurons (Fig. 1D). A time of 2 weeks was chosen to allow recovery of motoneurons which may have undergone any local axonal damage during the injection procedure. Approximately 18 μl of tracer was delivered over the course of 5 minutes to each head of the gastrocnemius muscle using a 26-gauge needle connected to a Hamilton microsyringe. The needle was advanced across the belly of the muscle and withdrawn slowly during the injection to leave a track of injected material across the entire width of the muscle. After careful retraction of the syringe to avoid leakage of fluororuby, the injection site was cleaned and the skin sutured. Animals were perfused as postfixed as described above, and the spinal cords were cut in 40 μm cross sections. Sections through the TIB motoneuronal pool were identified with fluorescence microscopy and every fifth section was kept for Nissl staining to accurately reflect the effects of increasing doses of BDNF on the entire TIB motoneuronal pool. The 40 μm cross sections were mounted on glass slides and stained with toluidine blue (0.5%). Light microscopic images of Nissl stained motoneurons were captured using a Sony LCD camera, and analyzed using JAVA video analysis software (Jandel Scientific, Corte Madera, CA). Motoneurons on the right and left sides were counted and cell body size was calculated to determine the effects of increasing doses of

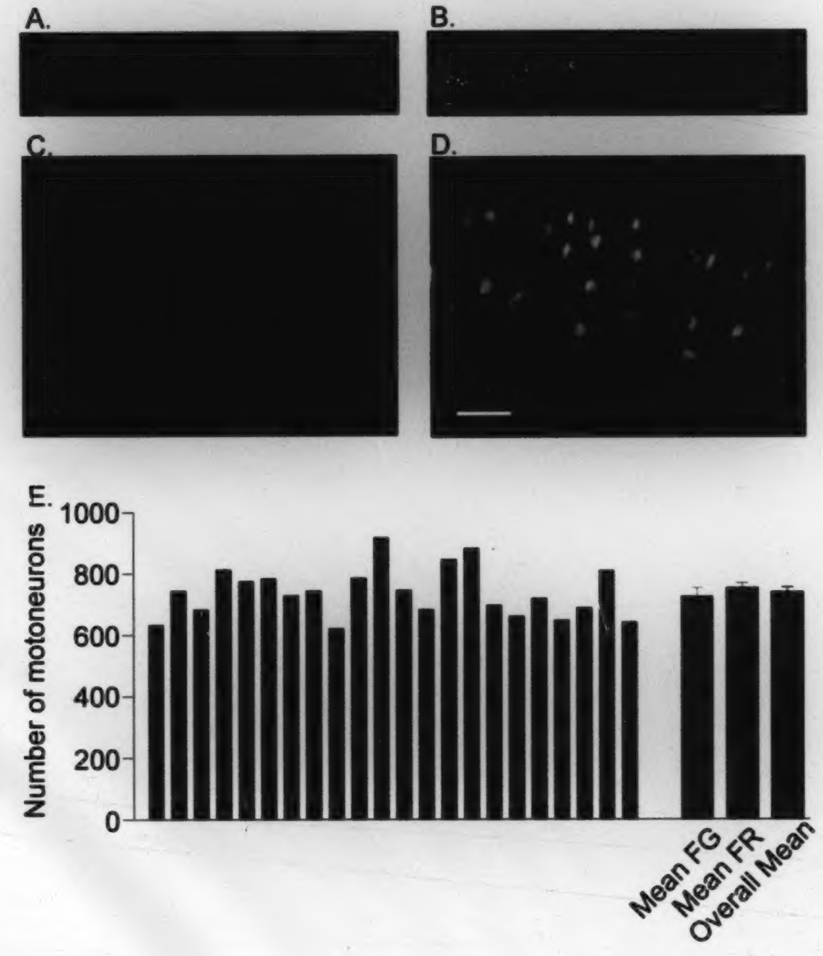
BDNF on motoneuronal survival and cell body size after injury.

2.2.8: Statistics

Differences between groups were detected using independent T-tests. Statistical significance was accepted at the level of $p<0.05$. Values are presented as the mean ± SE.

Figure 2-2. Fluororuby and fluorogold labeling of intact TIB motoneurons. Low (*A,B*) and high (*C,D*) magnification photomicrographs of representative 50 µm longitudinal sections through the ventral horn of the T11-L1 spinal segments of fluororuby (*A,C*) or fluororuby (*B,D*) labeled intact TIB motoneurons. Scale bars represent 100 µm. *E*. Quantification of the number (mean ± SE) of fluorogold (blue bars) and fluororuby (red bars) labeled TIB motoneurons shows no significant difference in the efficacy of the two dyes in labeling motoneurons ($p>0.05$). The numbers were pooled to reflect an overall mean number of motoneurons in the intact TIB motoneuronal pool.

Figure 2-2: Effective and consistent retrograde labeling of tibial motoneurons by fluororuby and fluorogold



2.3: Results

2.3.1: Fluorescent labeling of intact TIB motoneurons

To identify the number of motoneurons in the intact tibial (TIB) motoneuron pool, and to characterize the efficacy of fluorogold and fluororuby retrograde neuronal tracing of intact and regenerating TIB motoneurons, we labeled the contralateral intact TIB nerve in 23 rats (12 fluorogold, 11 fluororuby) in which the ipsilateral TIB nerve was cross-sutured to the distal stump of a freshly cut CP nerve (Figure 2-1C). Both retrogradely transported fluororuby (Figure 2- 2A,C), and fluorogold (Figure 2- 2B,D) intensely stained the cytoplasm of the TIB motoneuronal cell bodies and dendrites in 50 μm longitudinal sections of the T11-L1 segments of the lumbar spinal cord. There was no significant difference between the mean number of motoneurons labeled with either fluorogold or fluororuby (724 ± 27 versus 752 ± 14, respectively, Figure 2-2E). Therefore, the total number of intact labeled TIB motoneurons was pooled together to represent an overall 737 ± 16 motoneurons present in the intact motoneuronal pool for comparison with the number of TIB motoneurons which regenerated after cross suture and continuous BDNF or saline control infusion. This number is slightly less than what has been reported previously for the number of motoneurons in the intact TIB motoneuronal pool (Swett et al., 1986), possibly due to different labeling techniques and/or our more stringent criteria (i.e. counting only motoneurons with clearly visible nuclei) for counting labeled motoneurons. The low variability (2.1%) between rats in the number of fluorogold and fluororuby labeled intact TIB motoneurons illustrates the reliability and consistency of our retrograde labelling technique (Figure 2-2E).

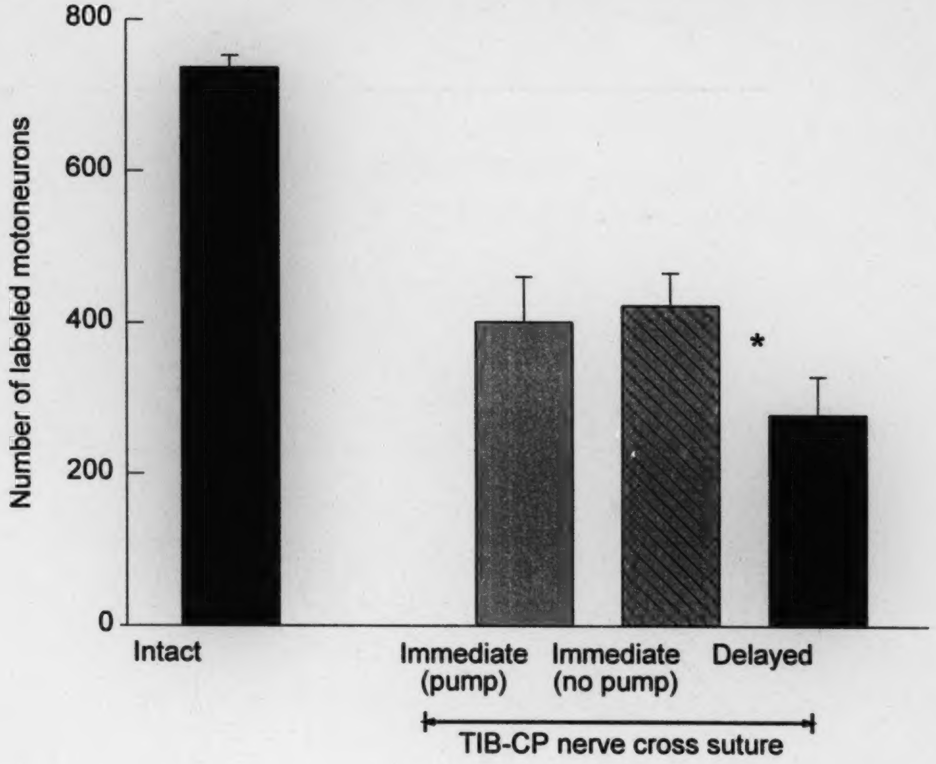
2.3.2: Chronic axotomy reduces TIB motor axonal regeneration

Motoneurons progressively lose the ability to regenerate their axons as a function of the duration of chronic axotomy prior to nerve repair (Fu and Gordon, 1995). Specifically, a 2 month period of chronic axotomy of posterior TIB motoneurons has been shown to reduce the number of reinnervated motor units in the denervated tibialis anterior muscle after cross-suture to a freshly cut distal CP nerve stump to 66% of the number of motor units counted after immediate nerve repair (Fu and Gordon, 1995). This 2 month period of chronic axotomy prior to cross suture to a freshly denervated CP distal nerve stump (Figure

2-1A,B) was selected in the present study because a reduction in regenerative capacity to 66% of immediate nerve repair would facilitate the detection of both positive and/or negative effects of exogenous BDNF on motor axonal regeneration. The mean number of fluorogold or fluororuby backlabelled TIB motoneurons which regenerated axons 20 mm from the site of TIB-CP cross-suture 4 weeks after immediate nerve repair was approximately half the total number intact TIB motoneurons backlabelled on the contralateral side (Figure 2-3). The low numbers of motoneurons which regenerate their axons in a 4 week period compared to the higher number of labeled intact motoneurons is consistent with the 8-10 weeks period required for all axons to regenerate across the surgical site (Al-Majed et al., 2000a).

Figure 2-3. *A*. Mean (± SE) number of tibial (TIB) motoneurons in the intact contralateral motoneuronal pool (black bar), and the significantly reduced number of motoneurons which regenerate their axons a distance of 20 mm in 28 days after immediate nerve repair and continuous saline administration via mini osmotic pumps (light grey bar) and in the presence of saline soaked gelfoam (striped bar). After delayed nerve repair, significantly fewer motoneurons regenerated their axons (dark grey bar).

Figure 2-3: Motor axonal regeneration is reduced by chronic axotomy, but not by the drug delivery hardware



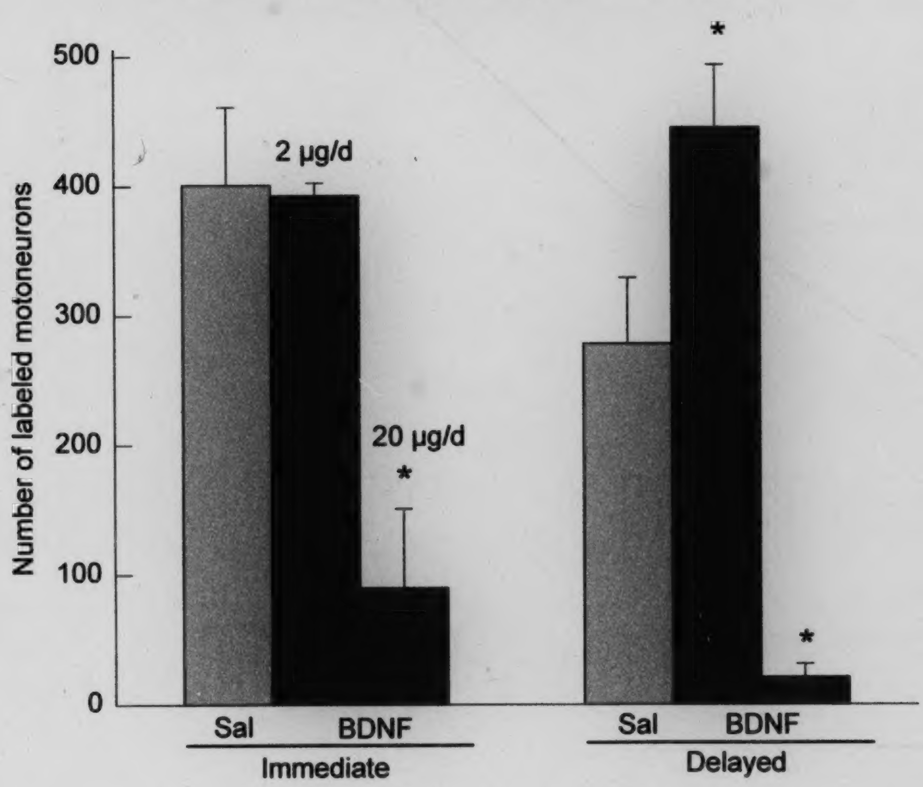
The number of motoneurons which regenerated their axons after chronic axotomy and delayed nerve repair was significantly reduced compared to immediate nerve repair (Fig. 2–3). The low number of chronically axotomized motoneurons which regenerated their axons reflects a reduction to 69% of the regenerative capacity after immediate nerve repair. This reduction is in excellent agreement with the previous findings of a reduction to 66% in number of reinnervated motor units after the same 2 month period of chronic axotomy (Fu and Gordon, 1995). These data demonstrate that chronic axotomy reduces the number of TIB motoneurons which regenerate their axons, thereby affirming the conclusions made by Fu and Gordon (1995) that chronic axotomy reduces the number of motor units which regenerate and reinnervate the denervated tibialis anterior muscle.

2.3.3: Delivery hardware does not impair axonal regeneration

A wide variety of experimental approaches have been used to locally administer neurotrophic factors to regenerating peripheral nerves, including gelfoam (Koliatsos et al., 1993), osmotic pumps with catheters anchored near the site of epineurial coaptation (Lewin et al., 1996; Utley et al., 1997), repair with collagen tubules embedded with neurotrophic factors (Ho et al., 1998), fibronectin mats impregnated with neurotrophic factors (Sterne et al., 1997ab, Simon et al., 2000), and injection into silicone channels (Shirley et al., 1996). However, this is the first study to use 4 week osmotic pumps to deliver neurotrophic factors directly to the site of injury and surgical repair (Figure 2-1B). To examine whether the drug delivery hardware used in this study had any effect on motor axonal regeneration, we compared the number of fluorescently labeled TIB motoneurons which regenerated their axons into a freshly cut CP distal nerve stump after immediate nerve repair and either 1) continuous saline administration via a 4 week osmotic pump or 2) exposure to a closely apposed 5 X 20 mm piece of saline soaked gelfoam. The number of motoneurons which regenerated their axons in a 4 week period after immediate nerve repair and exposure to saline soaked gelfoam was not significantly different from the number which regenerate when saline is administered via the osmotic pump (Figure 2-3). Thus the presence of the osmotic pump, nerve cuff, and drug delivery catheter did not interfere with the regeneration of the TIB motor axons.

Figure 2-4: Effects of exogenous BDNF on motor axonal regeneration after immediate and delayed nerve repair after chronic axotomy. The mean (± SE) number of TIB motoneurons which regenerated their axons after immediate nerve suture was not changed by low dose (2 μg/day) BDNF treatment, but significantly reduced by high dose (20 μg/day) BDNF treatment. After delayed nerve repair, the mean number of saline treated 2 month chronically axotomized motoneurons was significantly lower than immediate nerve suture. Low dose BDNF increased the number of TIB motoneurons which regenerated their axons to equal the number after immediate nerve repair, and high dose BDNF treatment drastically reduced TIB axonal regeneration. * - denotes $p<0.05$ relative to saline controls.

Figure 2-4: Effects of exogenous BDNF on motor axonal regeneration after both immediate nerve repair as well as nerve repair after chronic axotomy



2.3.4: Low dose BDNF (2 μg/day) has no effect on motor axonal regeneration after immediate repair, but reverses the negative effects of chronic axotomy

To examine the effects of exogenous BDNF on motor axonal regeneration, we counted the number of fluorescently labeled motoneurons which regenerated a distance of 20 mm into a freshly cut distal CP nerve stump in 4 weeks, after either immediate nerve repair, or delayed nerve repair 2 months after chronic TIB axotomy (Fig. 2-1C). Continuous local application of exogenous BDNF for the 4 week period of axonal regeneration, at low doses of 2 μg/day which were previously reported to promote the survival of axotomized neonatal motoneurons (Koliatsos et al., 1993), had no detectable effect on the number of TIB motoneurons which regenerated axons following immediate nerve repair as compared to the saline control (Figure 2-4). However, the same low dose significantly increased the number of 2 month chronically axotomized TIB motoneurons which regenerated axons after delayed TIB-CP cross suture, which represents a 182% increase compared to the saline control (Figure 2-4). The beneficial effect of low dose BDNF on axonal regeneration of chronically axotomized TIB motoneurons is clearly apparent in the representative photomicrographs of retrogradely labelled TIB motoneurons (Figure 5 A,B). Thus low dose BDNF restored the number of chronically axotomized motoneurons which regenerated axons to the number which regenerated after immediate TIB-CP cross suture (Figure 2-4). The robust axonal regeneration of chronically axotomized TIB motoneurons through the injury site and into the distal CP nerve stump after low dose BDNF treatment was evident in longitudinal sections of the TIB-CP repair site (Figure 2-6A). Therefore, low dose BDNF promotes axonal regeneration and completely reverses the negative effects of chronic axotomy.

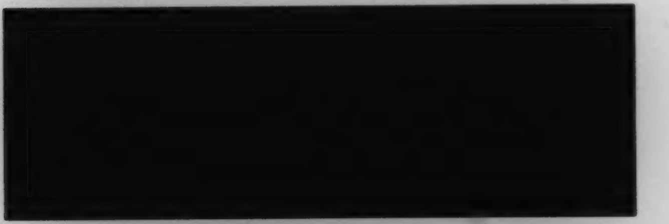
2.3.5: High dose BDNF (20 μg/day) inhibits motor axonal regeneration

Although BDNF has been shown to maintain the cholinergic phenotype in axotomized motoneurons by preventing the axotomy-induced decline in ChAT (Yan et al., 1992; Friedman et al., 1995; Tuszynski et al., 1996; Kishino et al., 1997), a direct link between ChAT expression and motor axonal regeneration has not yet been demonstrated. To examine the effects of higher doses of BDNF previously shown to prevent the decline in motoneuronal expression of ChAT, on motor axonal regeneration (12 and 60 μg/day;

Figure 2-5: Bimodal effects of exogenous BDNF on the number of fluorsecently labeled TIB motoneurons. Low power photomicrographs of representative 50 µm longitudinal sections through the ventral horn of the T11-L1 spinal segments of 2 month chronically axotomized TIB motoneurons which regenerated their axons and were backlabelled with a fluorescent retrograde neurotracer following saline (*A*), low dose BDNF (*B*), or high dose BDNF (*C*) treatment. Scale bar represents 200 µm.

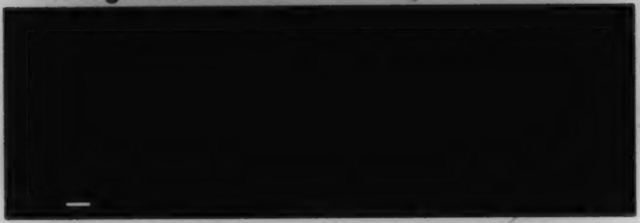
Figure 2-5: Low dose exogenous BDNF reverses the negative effect of chronic axotomy but high dose exogenous BDNF reduces the number of motoneurons which regenerate their axons

A. Saline



B. Low Dose BDNF

C. High Dose BDNF

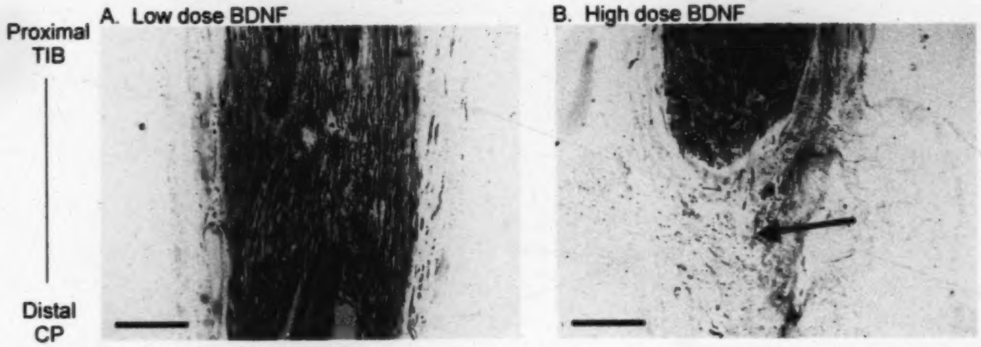


Friedman et al., 1995, Kishino et al., 1997), we counted motoneurons which regenerated axons after exogenous application of the high dose of 20 µg/day BDNF on the TIB-CP cross-suture site. High dose BDNF profoundly reduced the number of TIB motoneurons whcih regenerated after immediate nerve repair to 90 ± 61, a decrease to 22% of the immediate nerve repair saline control (Figure 2-4), which is evident in the photomicrographs of representative longitudinal sections through the TIB motoneuronal pool (Figure 2-5 A, C). Moreover, in contrast to the facilitatory effect of low dose BDNF (2 µg/day), the dramatic effect of high dose BDNF in reducing the number of motoneurons which regenerated their axons is shown in Figure 2-6. The mean number of chronically axotomized motoneurons which regenerated their axons was 21 ± 10, an even larger decline to 8% of the delayed nerve repair saline control (Figure 2-4).

One possibility is that the negative effects of high dose BDNF on numbers of backlabelled motoneurons may be accounted for by an impairment of retrograde transport of the fluorescent neurotracers used to evaluate axonal regeneration. This possibility is not likely in view of the data from Sagot et al. (1998) demonstrating that the application of the same high doses of BDNF to the proximal sciatic nerve stump had no effect on the retrograde transport of fluorogold and in turn, the number of backlabelled sciatic motoneurons. The inhibitory effects of high dose BDNF on the number of motoneurons which regenerated axons are supported by observations of dramatically reduced numbers of regenerated axons through the injury site (Figure 2-6 B) into the distal stump, as compared to the low dose BDNF treated axons (Figure 2-6 A), using a modified Bielschowsky's stain of the axons in longitudinal sections of the TIB-CP suture site.

Figure 2-6: Visualization of regenerated TIB axons is consistent with the number of fluorescently labeled TIB motoneurons. Photomicrographs of representative 8 μm longitudinal sections of the TIB-CP nerve repair sites stained with Bielschowski's method shows substantial axonal regeneration through the injury site and into the distal CP nerve stump after 28 days low dose (*A*), but not high dose (*B*) BDNF (arrow). Scale bar represents 300 μm.

Figure 2-6: The number of TIB axons that cross the injury site is consistent with the number of fluorescently labeled motoneurons

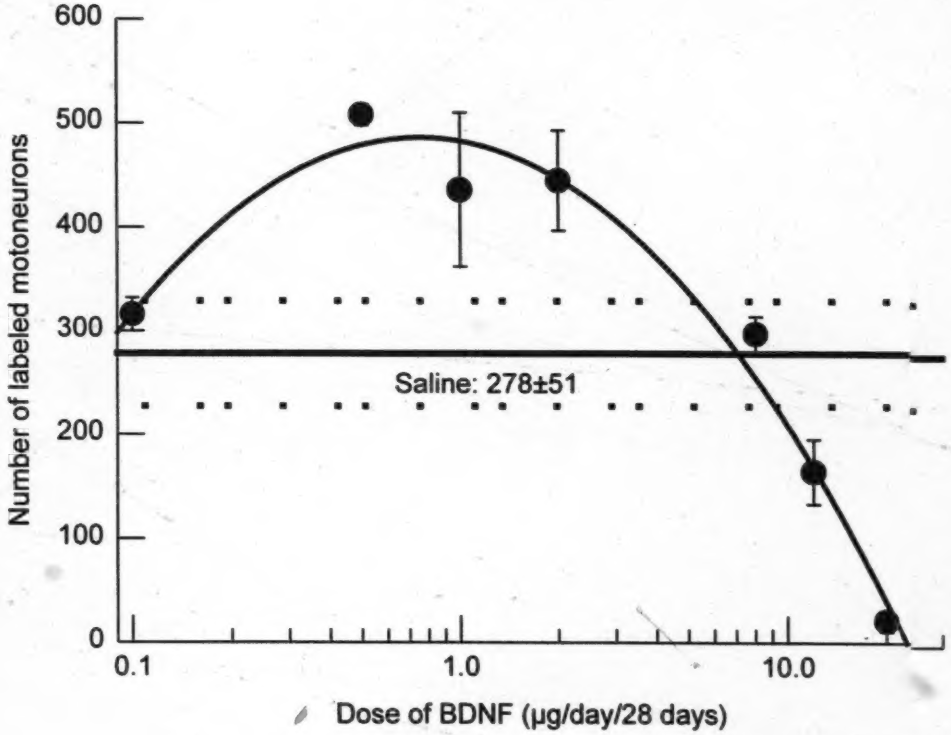

A. Low dose BDNF

B. High dose BDNF

Proximal
TIB
Distal
CP

Figure 2-7. Bimodal regulation of motor axonal regeneration by exogenous BDNF. Dose-response relationship between the number of 2 month chronically axotomized tibial (TIB) motoneurons which regenerate axons into freshly denervated common peroneal nerve stumps and the daily dose of exogenous BDNF compared with saline administration. Low doses of BDNF (0.5-2 μg/day) significantly promote motor axonal regeneration compared to saline controls (horizontal reference lines represent saline mean ± SE). In contrast, doses above 8 μg/day significantly inhibit motor axonal regeneration in a dose-dependent fashion.

Figure 2-7: Dose-dependent facilitatory and inhibitory effects of exogenous BDNF on motor axonal regeneration after after a 2 month period of chronic axotomy



2.3.6: BDNF's enhancement and inhibition of motor axonal regeneration is dose dependent

To further explore the bimodal facilitatory and inhibitory effects on motor axonal regeneration, we expanded the range of doses administered to chronically axotomized motoneurons to include 0.1, 0.5, 1, 8 and 12 μg/day in addition to the 2 and 20 μg/day doses described above. A minimum of 3 animals were examined at each dose. We counted the number of fluorescently labeled TIB motoneurons which regenerated their axons into a freshly cut CP distal nerve stump after a 2 month period of chronic axotomy. At the lowest dose of 0.1 μg/day, BDNF did not significantly improve axonal regeneration above the saline controls (Figure 2-7; horizontal reference line represents the saline treated mean ± SE). As the dose was increased to 0.5 μg/day, motor axonal regeneration was significantly increased, almost doubling the number of motoneurons which regenerate their axons in the saline control group. However, at doses of BDNF higher than 2 μg/day, there was a steep dose-dependent decrease in the number of labeled motoneurons, diminishing to less than 8% of the saline control at a dose of 20 μg/day, reflecting a 92% inhibition of motor axonal regeneration compared to saline controls. Hence, there is a biphasic dose-response relationship between exogenous BDNF and the number of chronically axotomized motoneurons which regenerate their axons into a freshly denervated distal nerve stump. In light of a time dependent decline in the biological activity of very high doses of BDNF (3-100x the highest dose of exogeneous BDNF used in our study) to a plateau level of 63% within 15-21 days (Dittrich et al., 1996), the dose of the exogenous BDNF shown on the x-axis progressively overestimates the biologically active BDNF concentration as a function of time over the 28 day period of administration. Hence, the doses of exogenous BDNF with profound inhibitory effects on axonal regeneration are lower than those shown in Figure 2-7. Furthermore, the potent inhibitory effects of high dose BDNF cannot be explained simply by a more rapid decline in biological activity for the higher doses, as this would shift the number of labelled motoneurons to the left of the dose-response curve such that, compared to saline controls, more motoneurons would regenerate their axons, not less. In other words, as the biological activity of BDNF decreases, the number of motoneurons which regenerated their axons would approach levels similar to our saline controls, not 92% less. Thus, there is a bimodal effect of exogenous BDNF on axonal regeneration which is dose-dependent which cannot be explained by reduced biological activity. However, it is important to exclude any possibilities that exogenous application of BDNF is neurotoxic.

2.3.7: Exogenous BDNF reverses soma atrophy and is not neurotoxic

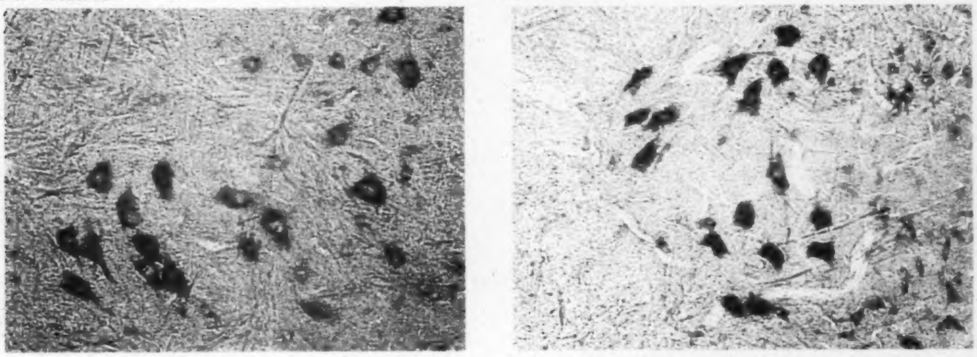
In light of findings from 3 previous studies, it is unlikely that the low numbers of labeled motoneurons which regenerated their axons after administration of high doses of exogenous BDNF (12-20 μg/day) are accounted for by a neurotoxic effect of the BDNF. Four weeks of local infusion of extremely high doses of NT-4/5 (60 μg/day), which also binds to trkB and p75 receptors, showed a beneficial effect of promoting the recovery of conduction velocity in axotomized TIB motoneurons, without any detectable signs of toxicity (Munson et al., 1997). In a study of survival of adult sciatic motoneurons after ventral root avulsion, 12 μg/day BDNF administered by Alzet mini-osmotic pumps over a 28 day period, significantly increased the number of surviving motoneurons (Kishino et al., 1997). Doses of exogenous BDNF of 60 μg/day maintained the expression of ChAT in axotomized sciatic motoneurons also without any apparent toxicity (Friedman et al., 1995). In order to exclude any neurotoxic effects of exogenous BDNF in our study of axonal regeneration, we counted the number of Nissl positive motoneurons in rats which received low and high doses of BDNF for 4 weeks during axonal regeneration and compared this number to the number in saline controls. The injured TIB motoneuronal pool was identified by retrograde labeling of the contralateral intact TIB motoneuronal pool from the left gastrocnemius muscle (Figure 2-1D). Nissl positive intact and axotomized motoneurons on the left and right ventral horns of the lumbar spinal cord were counted in every fifth 40 μm cross section of the spinal cord (Figure 2-8 A-D). As shown in Figure 2-9 A-C, the rostro-caudal distribution of motoneurons was similar, and the number (± SE, n=6 per group) of Nissl positive motoneurons present in the TIB motoneuronal pool was not significantly different for saline, low dose BDNF (2 μg/day), and high dose BDNF (20 μg/day) treated motoneurons (Figure 2-9 D).

Figure 2-8. Exogenous BDNF has no effect on number of 2 month chronically axotomized tibial (TIB) motoneurons. Photomicrographs of representitive Nissl positive intact (*A*) and axotomized TIB motoneurons treated with saline (*B*), low dose (2 μg/day; *C*), and high dose (2 μg/day; *D*) BDNF in 40 μm cross sections through the T11-L1 segments of the spinal cord. Scale bar represents 100 μm.

Figure 2-8: Exogenous BDNF does not affect the number of motoneurons in the T11-L1 spinal segments

A. Intact

B. Saline



C. Low dose BDNF

D. High dose BDNF

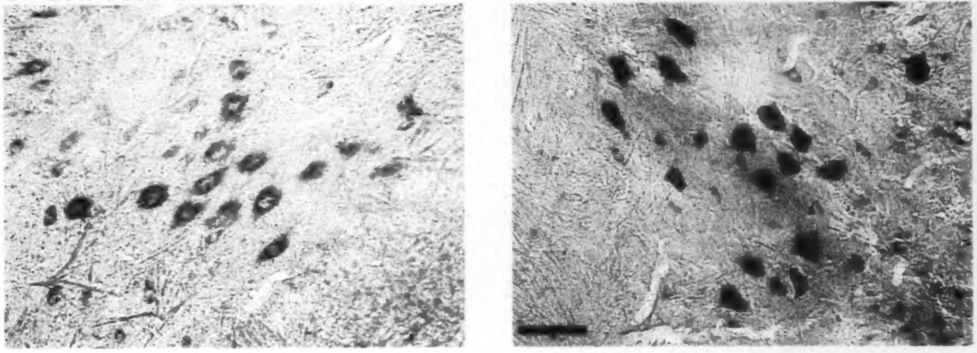
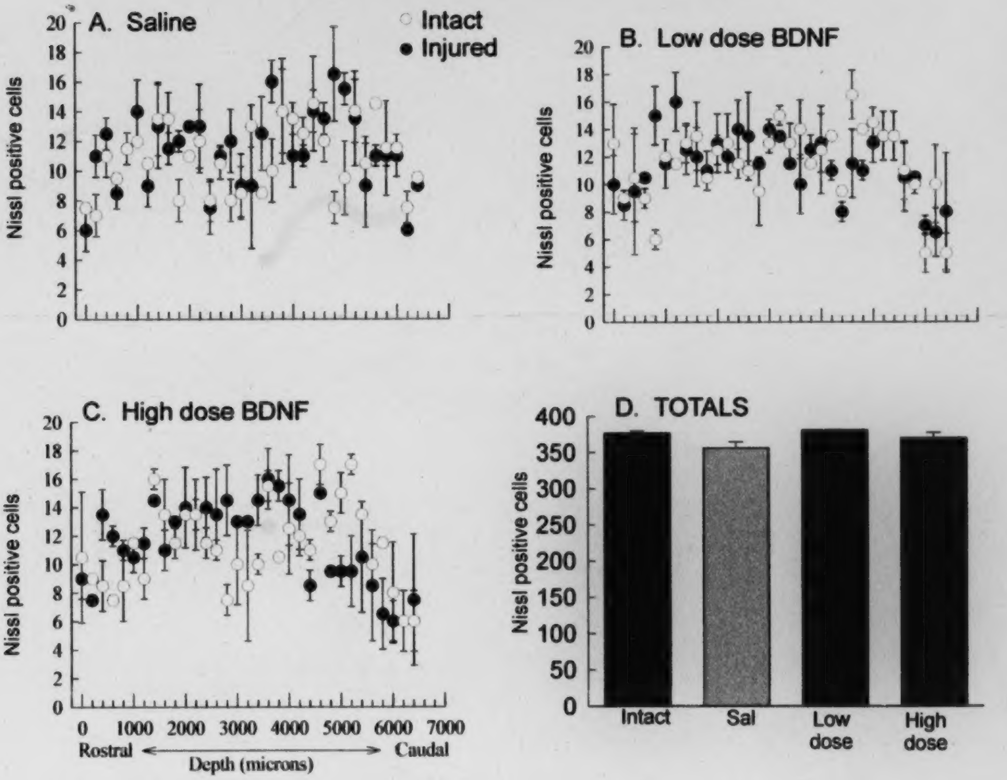


Figure 2-9: Quantification of the number of Nissl-positive motoneurons in the T11-L1 spinal segments. Mean (± SE) number of intact and injured (2 month chronic axotomy) Nissl positive TIB motoneurons as a function of rostral-caudal depth in the spinal cord of saline (*A*), low dose (*B*), and high dose (*C*) BDNF treated rats. *D*. Total (± SE) number of Nissl positive motoneurons across all groups was not statistically significant ($p > 0.05$)

Figure 2-9: Quantification of the number of Nissl positive cells in the T11-L1 spinal segments demonstrates that exogenous BDNF does not affect the survival of chronically axotomized motoneurons



From these studies, we conclude that high dose BDNF does not reduce numbers of labeled motoneurons by exerting a neurotoxic effect. Adult motoneurons rarely die after axotomy at sites distant from their cell bodies, and remain viable even after prolonged separation from the target (Gordon et al., 1991; Vanden Noven et al., 1993) in contrast to neonatal motoneurons which are highly susceptible to axotomy-induced cell death (Greensmith & Vrbova, 1996). Exogenous BDNF did not counteract this survival.

Photomicrographs of Nissl positive neurons were analyzed for cell body total area using JAVA image analysis software. A minimum of 100 cells was analyzed per treatment group. There was a significant decrease in the soma size of chronically axotomized TIB motoneurons which regenerated for a 4 week period in the presence of continuous saline infusion as compared to intact contralateral TIB motoneurons (Figure 2-10 A,B,E). Low, but not high dose BDNF treatment restored the mean cell soma size (Figure 2-10 C,D,E). Therefore, the positive effect of low dose BDNF in promoting motor axonal regeneration after a 2 month period of chronic axotomy is correlated with the ability of the same dose to reverse the atrophic changes in axotomized and regenerated motoneurons. In contrast, the strong inhibitory effects of high dose BDNF on motor axonal regeneration compared to saline controls could not be attributed to atrophy of motoneurons, as there was no significant difference in the cell body area between saline controls and high dose BDNF (Fig. 2-10 B,D,E).

2.3.8: p75 mediates inhibitory effects of high dose BDNF

Both trkB and p75 receptors are upregulated in motoneurons after axotomy (Ernfors et al., 1989; Funakoshi et al., 1993; Kobayashi et al., 1996; Hammarburg et al., 2000). In light of recent *in vitro* evidence for a role of p75 in mediating the inhibition of sympathetic neurite outgrowth (Kohn et al., 1999), we tested the possibility that binding of the exogenous BDNF to p75 receptors on the axotomized motoneurons mediates the inhibitory effects of high dose BDNF on axonal regeneration. We simultaneously infused the function blocking p75 antibody, REX, together with high dose (20 μg/day) BDNF either after immediate or delayed nerve repair to a freshly cut distal CP nerve stump. As shown in a representative photomicrographs of FR backlabelled TIB motoneurons in Figure 2-11A and B and in the

Figure 2-10. Chronic axotomy-induced atrophy of tibial (TIB) motoneurons is reversed by low, but not high dose BDNF. *A-D*. High power photomicrographs of Nissl positive cells in representative 40 μm cross sections through the T_{11}-L_1 spinal segments which contains the tibial (TIB) motoneuronal pool showing either intact (*A*) and (*B-D*) chronically axotomized motoneurons which have regenerated their axons into a freshly denervated common peroneal distal nerve stump. *E*. The mean (± SE) soma area of the intact (black bar) and axotomized saline and BDNF treated TIB motoneurons are compared. Saline treated axotomized TIB motoneurons were significantly smaller (grey bar). This reduction was reversed by low dose (red bar), but not high dose (blue bar) BDNF. * - denotes $p<0.05$.

Figure 2-10: Low, but not high dose BDNF prevents motoneuronal soma atrophy after a 2 month period of chronic axotomy

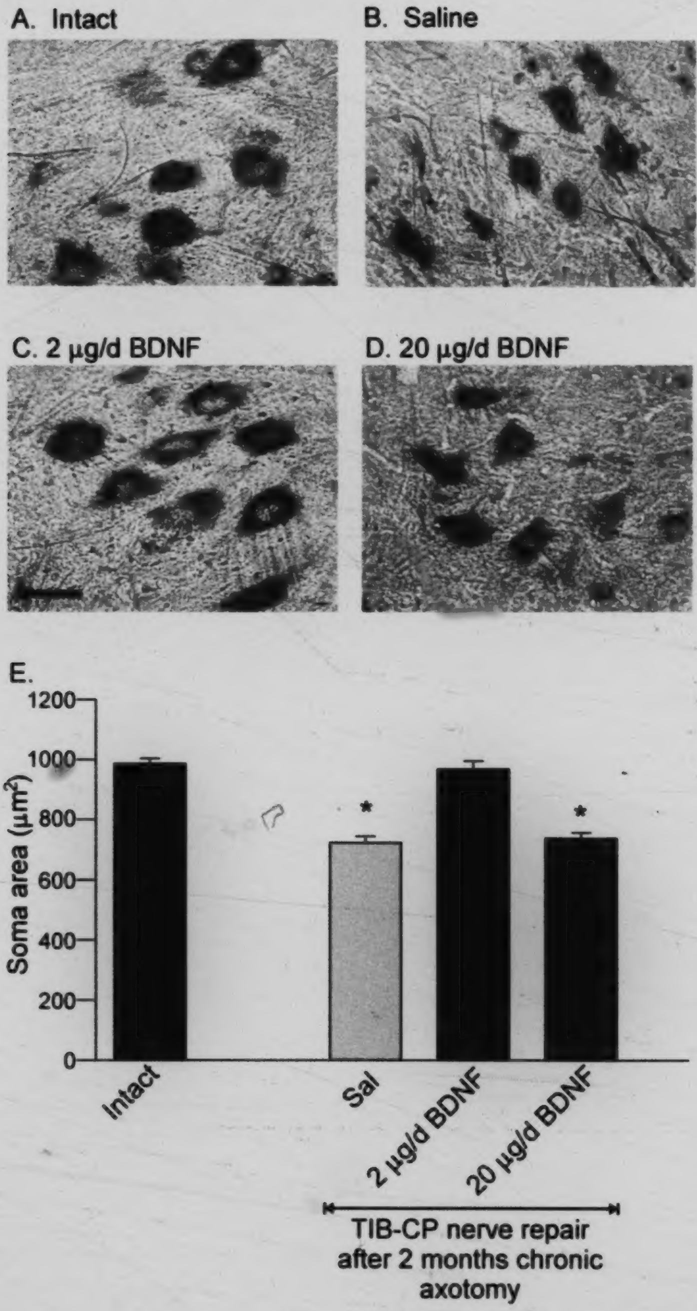
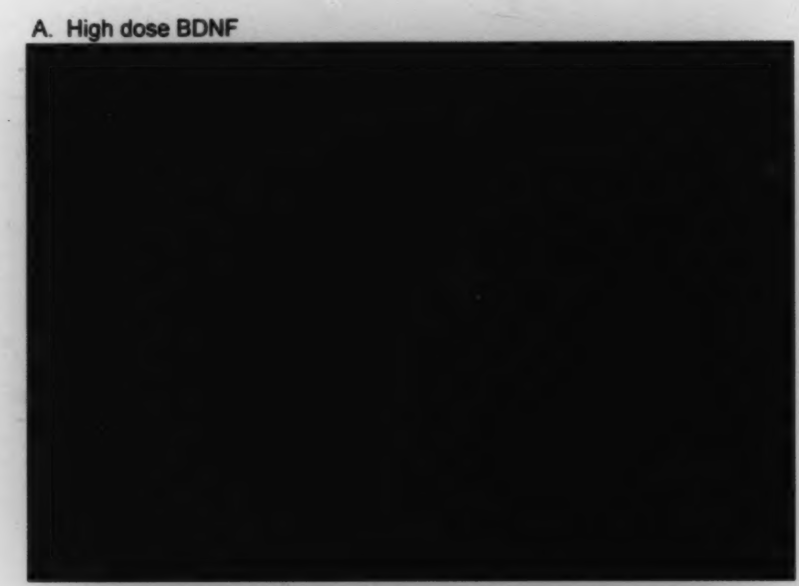


Figure 2-11. Inhibitory effects of exogenous high dose BDNF are reversed by preventing binding to p75 receptors with REX. Photomicrographs of fluororuby labeled tibial (TIB) motoneurons which have been treated with (*A*) high dose BDNF (20 μg/day) or (*B*) high dose BDNF + anti-p75 antibody (20 μg/day + 1:100 REX). Scale bar represents 100 μm.

Figure 2-11: Inhibitory effect of high dose exogenous BDNF on the number of motoneurons which regenerate axons is mediated by binding to p75 receptors

A. High dose BDNF



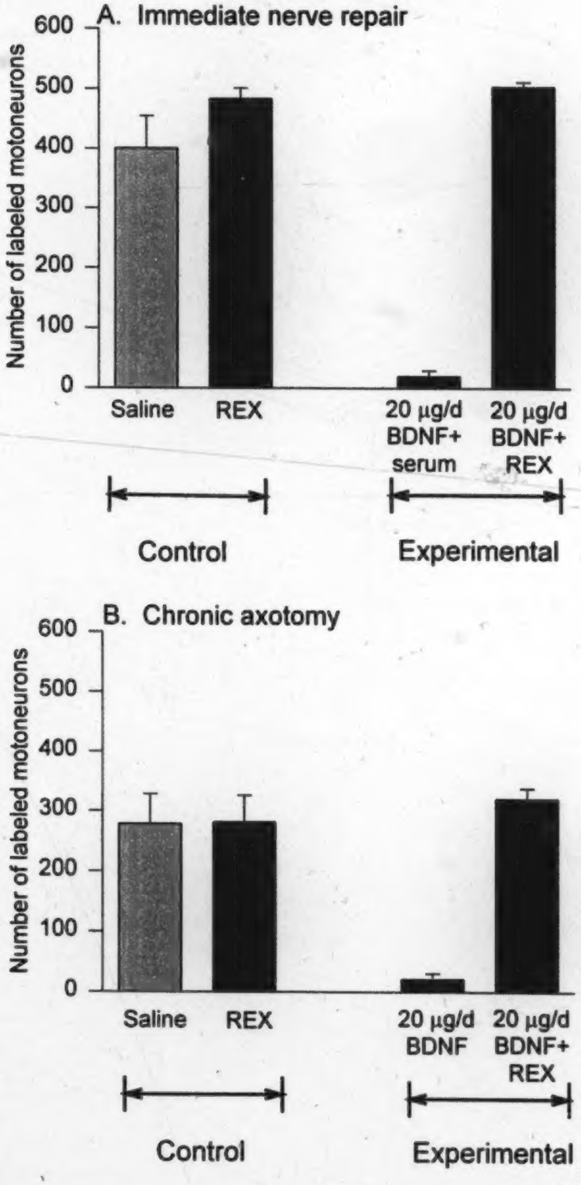
B. High dose BDNF + anti-p75 antibody

histograms in Figure 2-12 A and B, the anti-p75 antibody, REX, completely reversed the inhibitory effect of high dose BDNF, as compared to saline controls, in reducing the mean number of labeled motoneurons which regenerate their axons after immediate and delayed nerve repair.

The reversal of the inhibitory effects of high dose BDNF is not due to any non-specific actions of the REX antibody because the negative control administration of normal rabbit serum with high dose BDNF at the same concentration as the p75 REX antiserum did not reverse the inhibitory action of high dose BDNF on motor axonal regeneration after immediate nerve repair (Figure 2-12 A). Experiments with the control serum were not repeated for nerve repair after chronic axotomy. Finally, we demonstrated that REX had no effect on numbers of motoneurons which regenerated their axons after both immediate and delayed nerve repair (Figure 2-12 A,B). This shows that the p75-antibody, by preventing the binding of the high dose exogenous BDNF, is directly responsible for reversing the inhibitory action of the high dose BDNF on motor axonal regeneration.

Figure 2-12. The p75 receptor mediates the inhibitory effect of high dose BDNF. *A.* Mean (± SE) number of TIB motoneurons which regenerate a distance of 20 mm in 28 days following immediate nerve repair in the presence of saline (light grey bar), REX alone (dark grey bar), high dose BDNF + control serum (black bar), or high dose BDNF + REX (striped bar). Simultaneous application of high dose BDNF and REX completely reverses the inhibitory effects of high dose BDNF (striped bar), whereas control serum (black bar) did not. REX alone (dark grey) had no significant effect compared to the saline control ($p>0.05$). *B.* Mean (± SE) number of TIB motoneurons which regenerate a distance of 20 mm in 28 days following delayed nerve repair in the presence of saline (light grey bar), REX alone (dark grey bar), high dose BDNF (black bar), or high dose BDNF + REX (striped bar). Simultaneous application of high dose BDNF and REX completely reverses the inhibitory effects of high dose BDNF (grey bar). REX alone (black bar) had no significant effect compared to the saline control ($p>0.05$).

Figure 2-12: The p75 receptor mediates the inhibitory effect of high dose exogenous BDNF on the number of TIB motoneurons which regenerate their axons



2.4: DISCUSSION

This study provides the first direct and quantitative assay of the capacity of exogenous neurotrophic factors to influence axonal regeneration. We demonstrate a biphasic effect in which low doses of exogenous BDNF increase the number of chronically axotomized motoneurons which regenerate their axons into freshly denervated distal nerve stumps and higher doses progressively reduce the number. The inhibitory effects of high dose BDNF were seen also in freshly axotomized motoneurons which regenerated their axons. The inhibitory effects of BDNF on motor axonal regeneration after both immediate and delayed nerve repair were eliminated by blocking p75 receptors with function blocking antibodies.

2.4.1: Low dose BDNF promotes axonal regeneration after chronic axotomy

There have been previous reports of small improvements in functional recovery with exogenous application of BDNF but the evaluations of regeneration were both indirect and non-quantitative (Lewin et al., 1996; Utley et al., 1997; Moir et al., 2000). Interestingly in this regard, exogenous BDNF did not significantly improve motor axonal regeneration after immediate nerve repair (Figure 2-4). The number of saline treated TIB motoneurons which regenerated 20 mm after immediate TIB-CP cross suture reached ~55% of the total within 4 weeks of the suture (Figure 2-3). This is consistent with the findings of Al-Majed et al. (2000a) of a staggered axonal regeneration in which the same percentage (~55%) of cut and sutured femoral motoneurons regenerate their axons over the same distance within 4 weeks, the remaining axons regenerating over the following 4-6 weeks. The fact that exogenous BDNF did not increase the number above 55% indicates that BDNF does not accelerate axonal outgrowth across the surgical site. In contrast, the positive effects of low doses of BDNF of increasing the number of chronically axotomized motoneurons which regenerate their axons (Figure 2-4) and of reversing the somal atrophy of the motoneurons (Figure 2-9) indicate a role for BDNF in counteracting the progressive atrophy and failure of chronically axotomized motoneurons to regenerate their axons. It is conceivable therefore, that the early upregulation of BDNF in axotomized (Funakoshi et al., 1993; Kobayashi et al., 1996) and regenerating (Al-Majed et al., 2000b) motoneurons, is sufficient to sustain initial axonal growth, but the transient nature of this response may be one explanation for reduced axonal regeneration and functional recovery after chronic axotomy (Fu & Gordon, 1995).

Upregulation of growth associated genes, including tubulin, actin and GAP-43 in addition to BDNF, is also transient in axotomized motoneurons, the expression of the genes falling with time (Tetzlaff et al., 1991). Although these genes undergo a secondary upregulation in response to a refreshment injury, their expression is even more transient thereafter (Petrov et al., 1996). Application of exogenous BDNF may sustain the expression of these genes in the axotomized motoneurons as previously demonstrated in axotomized rubrospinal neurons (Kobayashi et al., 1997). The sustained expression of growth associated genes could, in turn, account for the positive effects of BDNF of promoting axonal regeneration of chronically axotomized motoneurons.

It has been recently demonstrated that NT-3 embedded in fibronectin mats which bridge a 10 mm gap between proximal and distal sciatic nerve stumps promotes axonal regeneration and selective reinnervation of fast fatigable muscle fibres which selectively express the myosin heavy chain type 2b (Sterne et al., 1997a,b). This is associated with better reinnervation of neuromuscular junctions in the fast extensor digitorum longus muscle, but not the slow soleus muscle (Simon et al., 2000). It remains to be determined whether the beneficial effect of low dose BDNF on motor axonal regeneration is associated with reinnervation of specific muscle types. However, it is an enticing possibility that different neurotrophic factors promote motor axonal regeneration and reinnervation of denervated muscles in a type-specific manner.

2.4.2: High dose BDNF inhibits axonal regeneration

Paradoxically, the BDNF dose of 12 μg/day which sustained expression of regeneration associated genes in the rubrospinal neurons (Kobayashi et al., 1997) profoundly inhibited TIB motor axonal regeneration in the present study (Figs 2-5 and 2-7). This apparent paradox may be reconciled by the important differences in expression of receptors for BDNF in the rubrospinal neurons and motoneurons in the central and peripheral nervous systems, respectively. Both sets of neurons express trkB after axotomy (Funakoshi et al., 1993; Kobayashi et al., 1996, 1997). In contrast, all the axotomized motoneurons also express p75 receptors, but only a fraction (<10%) of the rubrospinal neurons express p75 (Ernfors et al., 1989; Kobayashi et al., 1997). The contrasting expression levels of trkB and p75 in these neurons suggests that it may be the trkB receptors which mediate the positive effects of BDNF, and it is the p75 receptors which mediate the inhibitory effects of high dose BDNF on axonal regeneration of axotomized motoneurons. The positive effect of low dose exogenous BDNF on motor axonal regeneration being mediated by binding to trkB receptors is compatible with the critical importance of functional trkB receptors in preventing axotomy-induced cell death in neonatal motoneurons (Alcantara et al., 1997). Furthermore, adenoviral transfection of axotomized hypoglossal motoneurons with the serine threonine kinase Akt, which is activated in response to BDNF binding to trkB *in vitro* (Dolcet et al., 1999; Atwal et al., 2000), increased their rate of motor axonal regeneration (Namikawa et al., 2000). In contrast, the inhibitory effect of high dose exogenous BDNF being mediated by p75 receptors is consistent with recent work by Kohn et al. (1999) which demonstrated that NGF binding to its cognate trkA receptor promoted neurite outgrowth of sympathetic neurons *in vitro*, whereas application of BDNF inhibited this growth. It was presumed that BDNF mediated this inhibitory effect by binding to p75 receptors, since sympathetic neurons do not express trkB. This inhibitory effect on neurite outgrowth was reversed by preventing BDNF binding to p75 receptors with the same anti-p75 antibody used in this study.

2.4.3: Possible mechanisms for the bimodal regulation of motor axonal regeneration by BDNF

It has been also suggested that neuronal p75 receptors serve to restrict axonal growth and aid in pathfinding, since a combined p75 null mutant and NGF overexpressing mouse exhibits extensive sympathetic fibre growth into the white matter tracts of the CNS (Walsh et al., 1999). Contrary evidence for reduced axonal growth in embryonic p75 (-/-) knockout mice (Yamashita et al., 1999) may be explained by the fact that the physiological role of neuronal p75 receptors may differ depending on cell type and developmental stage (Wiese et al., 1999). Furthermore, the indirect evidence that there are more regenerated motor axonal profiles in adult p75 knockout mice than in wild type (Ferri et al., 1998) provides further indication that the inhibitory effects of high doses of exogenous BDNF on motor axonal regeneration may be mediated via the p75 receptors.

Isolated embryonic and neonatal motoneurons from mice p75 knockout mice are less responsive to BDNF than wild-type controls, suggesting that the expression of p75 on these cells is necessary for the formation of a high affinity receptor complex for BDNF (Wiese et al., 1999). Thus it is possible that co-infusion of the anti-p75 antibody with high dose BDNF reduces high affinity binding for BDNF in our model, and thereby would shift the entire BDNF dose response curve in Figure 2-7 to the right. However, it is difficult to extrapolate the results of an *in vitro* investigation of survival of isolated embryonic and neonatal motoneurons to an investigation of adult motor axonal regeneration, as these processes may be regulated by different mechanisms. Nonetheless, increasing doses of BDNF exert a dose-dependent facilitatory and inhibitory effect on the survival of motoneurons isolated from wild-type mice, but this inhibitory effect of higher doses of BDNF was eliminated in p75 (-/-) knockout motoneurons (Wiese et al., 1999). These results add support to our hypothesis that there is a dose-dependent interaction between exogenous BDNF and its receptors which mediate the biphasic facilitatory and inhibitory effects on motor axonal regeneration.

It has also been demonstrated that chronic exogenous BDNF treatment can effectively reduce the available trkB binding sites and downregulate trkB mRNA *in vitro* (Carter et al., 1995, Frank et al., 1996), and trkB protein *in vivo* (Frank et al., 1997). Thus motoneurons may be desensitizing with respect to trkB receptors in response to BDNF in our model, especially at the high doses of BDNF. This would in fact, explain why the positive effects of BDNF are only seen at doses of between 0.5 and 2 μg/day (Figure 2-7), but returned to baseline at a dose of 8 μg/day. However, receptor desensitization alone would not explain the potent inhibitory effects of 12 and 20 μg/day. Consistent with trkB receptor downregulation, preventing the binding of high doses of exogenous BDNF to p75 receptors with the anti-p75 antibody reversed the inhibitory effects of high dose BDNF, but did not restore the facilitatory effects of BDNF on motor axonal regeneration after chronic axotomy seen at low doses (Figure 2-12 B). Nonetheless, it should be considered that, although trkB protein levels were decreased with chronic BDNF treatment, trkB mRNA remained unchanged and the physiological response to BDNF remained unattenuated *in vivo*, thus indicating that the reduced levels of trkB protein more likely reflect increased protein turnover rather than decreased trkB gene expression (Frank et al., 1997).

As there is reduced Schwann cell apoptosis in p75 knockout mice (Ferri and Bisby, 1999), the possibility exists that activation of the p75 receptors on the Schwann cells by exogenous BDNF decreases the numbers of Schwann cells to support axonal regeneration. Nonetheless, such toxic effects of BDNF on Schwann cells have not yet been demonstrated *in vivo*. In addition, *in vitro* studies have demonstrated that BDNF is not a potent activator of biological responses mediated by p75 in Schwann cells, such as the induction of NF-κB (Carter et al., 1996). Our findings that high dose BDNF inhibited the axonal regeneration of chronically axotomized, far more than freshly axotomized, motoneurons into a to a freshly denervated distal nerve stump (Figure 2-4) also strongly implicate the p75 receptors on the motoneurons, as opposed to the p75 receptors on Schwann cells of the distal nerve stump, as mediators of the strong inhibitory effects of high dose exogenous BDNF.

2.5: Conclusion

A possible functional significance of the inhibitory effects of high dose BDNF on motor axonal regeneration is that growth cones are exposed to high concentrations of several neurotrophic factors, including NGF, NT-3 and NT-4/5 upon arrival at denervated muscle (reviewed by Fu & Gordon, 1997). Inhibition of growth via the p75 receptor would serve to stop axonal growth. Stop signals at the neuromuscular junction such as S-laminin (Hunter et al., 1989, Noakes et al., 1995) together with growth inhibition via p75 may serve to initiate the formation of the neuromuscular synapse on denervated muscle fibers.

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Chapter 3: The neurotrophin receptors, trkB and p75, receptors differentially regulate motor axonal regeneration

3.1: Introduction

The neurotrophins are a family of neurotrophic factors which are structurally and functionally related peptides which mediate survival, differentiation, neurite outgrowth, and functional plasticity in a wide variety of neuronal populations in the central and peripheral nervous systems. In mammals, the neurotrophin family includes nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin (NT-3), and NT-4/5. Neurotrophins mediate their cellular effects by binding to two classes of membrane bound receptors, members of the tropomyosin receptor kinase (trk) family, and the p75 receptor, a member of the tumour necrosis factor family of receptors (reviewed in Yano & Chao, 2000). Whereas all neurotrophins bind p75 with similar affinity, the trk family of receptors are more specific; NGF binds trkA, BDNF and NT-4/5 bind trkB, and NT-3 binds to trkC and trkA, although to a lesser extent (Rodriguez-Tebar et al., 1992; Klein et al., 1991a,b; Squinto et al., 1991, Lamballe et al., 1991).

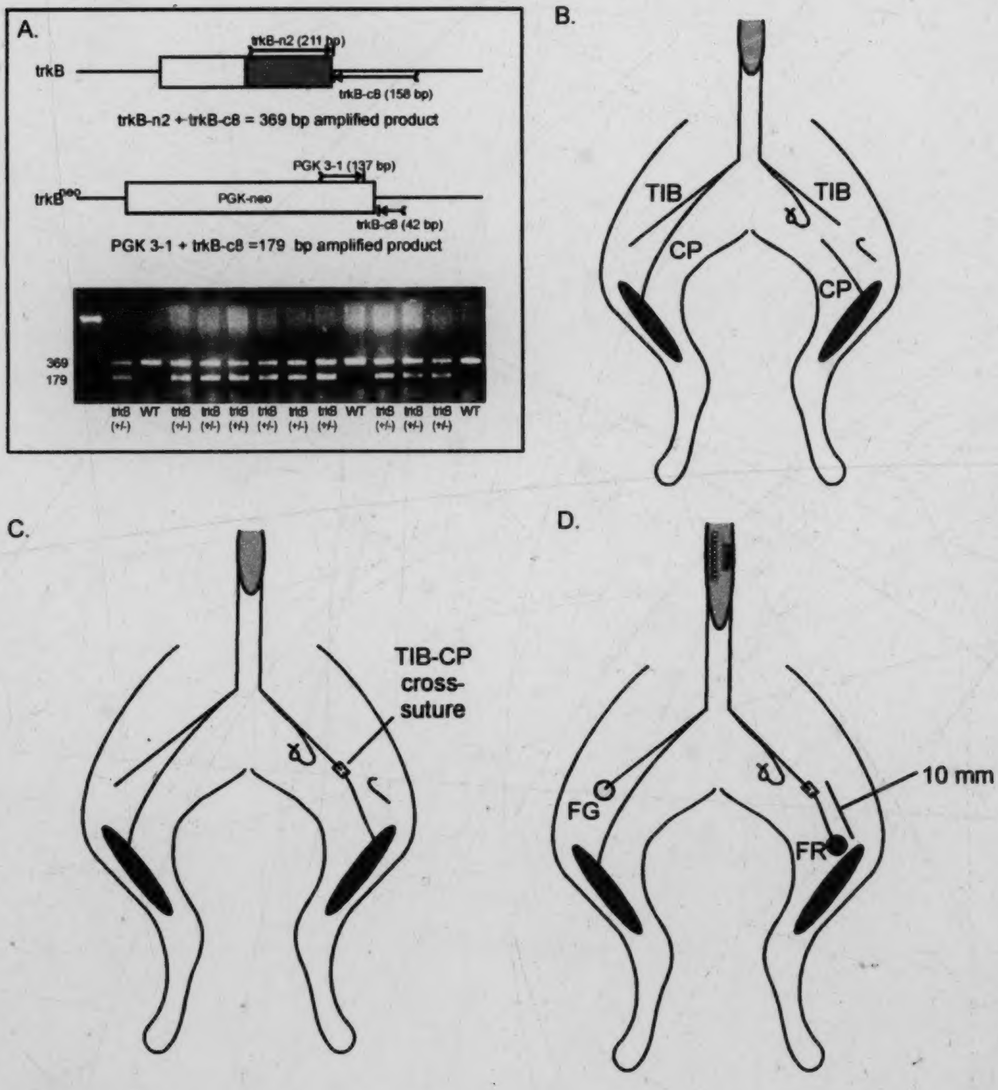
Based on neuronal expression of neurotrophins and their receptors after peripheral nerve injury, as well as their upregulation in the non-neuronal cells of the distal nerve stump, neurotrophins have been proposed to be involved in peripheral nerve regeneration. Specifically, a strong role has been suggested for BDNF in motor axonal regeneration, as axotomized motoneurons express BDNF, trkB, and p75 receptors after injury, and denervated Schwann cells of the distal nerve stump upregulate BDNF and p75 (Funakoshi et al., 1993; Kobayashi et al., 1996; Hammarburg et al., 2000; You et al., 1996). In addition, BDNF promotes the survival of axotomized motoneurons in many experimental models (Sendtner et al., 1992; Koliatsos et al., 1993; Yan et al., 1992; Vejsada et al., 1995; 1998; Novikova et al., 1997; Novikov et al., 1997; Kishino et al., 1997), and at low doses promotes the regeneration of chronically axotomized motoneurons (Chapter 2).

Despite our knowledge of the functionally distinct biological effects which are mediated by the two classes of neurotrophin receptors *in vitro* (Barrett, 2000; Friedman & Greene, 1999; Casaccia-Bonnefil et al., 1998; 1999a,b; Chao et al., 1998; Kaplan & Miller, 1997, 2000; Segal & Greenberg, 1996; Klesse & Parada, 1999; Barker, 1998; Yano & Chao, 2000; Dobrowsky & Carter, 1998), surprisingly little is known about the receptors involved in mediating neurotrophin effects *in vivo*. It is becoming apparent that trkB and p75 receptors differentially regulate survival of axotomized motoneurons. For example, trkB receptors are critically important in promoting the survival of axotomized neonatal motoneurons (Alcantara et al., 1997), whereas the expression of p75 receptors may amplify axotomy-induced motoneuronal cell death (Ferri et al., 1998). The latter effect is consistent with an inhibitory role of p75 on axonal sprouting both *in vitro* and *in vivo* (Walsh et al., 1999; Ferri et al., 1998; Kohn et al., 1999). In addition, we have previously demonstrated that exogenous BDNF exerts a biphasic dose-dependent facilitatory and inhibitory effect on motor axonal regeneration (Chapter 2). The latter effect of high dose BDNF are attributed to binding to p75 receptors, as the inhibitory effect is reversed by simultaneous application of a function blocking anti-p75 antibody.

To directly examine the role of trkB and p75 receptors in mediating the effects of endogenous BDNF and NT-4/5 on motor axonal regeneration, we have quantified the number of motoneurons which regenerate their axons over time in p75 (-/-) and trkB (+/-) knockout mice as well as wild type controls.

Figure 3-1. Identification of trkB (+/-) mice and schematic representation of animal surgery. *A.* TrkB (+/-) mice were identified on the basis of PCR analysis of a 179 bp amplified product isolated from an ear notch biopsy. PCR was performed by Ms. Jenn Whitmore of the University of Alberta - Health Sciences Laboratory Animal Services. *B.* The tibial (TIB) nerve was dissected and transected 5 mm distal to the trifurcation from the common peroneal (CP) and sural (not shown) branches of the sciatic nerve. A 5 mm segment of the TIB nerve was resected to prevent aberrant regeneration in the TIB distal nerve stump. *C.* The proximal TIB nerve stump was cross-sutured to a freshly denervated CP distal nerve stump within a 3 mm silastic nerve cuff. The proximal CP stump was sutured to the innervated biceps femoris muscle to prevent regeneration. *D.* Contralateral intact TIB motoneurons were labeled with fluorogold (FG), and axotomized TIB motoneurons which have regenerated their axons a distance of 10 mm were labeled with fluororuby (FR). Six days later, the animals were perfused and the number of FG labeled intact, and FR labeled TIB motoneurons which regenerated axons were counted in 50 μm longitudinal sections of the T11-L1 spinal segments.

Figure 3-1: Identification of trkB (+/-) mice and schematic representation of surgical procedures


A.
trkB
trkB-n2 (211 bp)
trkB-c8 (158 bp)
trkB-n2 + trkB-c8 = 369 bp amplified product
trkB^neo
PGK 3-1 (137 bp)
PGK-neo
trkB-c8 (42 bp)
PGK 3-1 + trkB-c8 =179 bp amplified product
369
179
trkB (+/-)
WT
trkB (+/-)
trkB (+/-)
trkB (+/-)
trkB (+/-)
trkB (+/-)
trkB (+/-)
WT
trkB (+/-)
trkB (+/-)
trkB (+/-)
WT
B.
TIB
TIB
CP
CP
C.
TIB-CP cross-suture
D.
FG
FR
10 mm

3.2: Methods:

3.2.1: Experimental animals:

To evaluate the role of trkB and p75 receptors in motor axonal regeneration, experiments were carried out in 4 strains of mice: i) p75 homozygous knockout mice (Jackson Labs, Bar Harbour, ME) which have been previously described (Lee et al., 1992), ii), C57Bl6-J mice, the background control strain for the p75 knockout mice, iii) trkB heterozygous knockout mice (a kind gift from Dr. Louis F. Reichardt, USCF), and iv) wild type C57Bl6-J littermates of the trkB heterozygous knockout mice. In all experiments, there were no differences between commercially available C57Bl6-J mice, and the wild type littermates of the trkB heterozygous knockout mice. Thus the data from these animals were pooled together to represent wild-type mice for comparisons with the p75 and trkB knockout mice

3.2.2: PCR Identification of trkB heterozygous knockout mice

The trkB heterozygous knockout mice breeding pairs were a generous gift from Dr. Louis F. Reichardt (USCF). At 4 weeks after birth, offspring were analyzed for expression of phosphoglycine kinase-neomyosin resistance (PGKneo) cassette which was inserted into the first coding exon of the trkB gene to disrupt both full length and truncated trkB isoforms (Xu, B, and Reichardt LF, unpublished observations). Wild type animals were identified on the basis of the detection of a 369 bp product amplified from the trkB primers5'-ATGTCGCCCTGGCTGAAGTG-3' (211 bp) and 3'-ACTGACATCCGTAAGCCA-5' (158 bp).TrkB mutant animals were identified on the basis of a 179 bp amplified product amplified from the PGK primer (PGK 3-1) 5'-GGTTCTAAGTACTGTGGTTTCC-3' (137 bp) and the trkB 3' primer described above (Figure 3-1A). Forty cycles of 94°C (1 min), 65°C (1.5 min), 72°C (1 min) were run in a standard thermal cycler. Samples were run on a 2% agarose gel.

Animals expressing only wild-type trkB were considered homozygous wild-type animals, whereas animals expressing both wild-type trkB, as well as the mutant trkB-PGKneo were considered heterozygous knockout mice (Figure 3-1A). No homozygous knockout mice were detected, as a homozygous trkB mutation confers embryonic lethality when expressed in a C57Bl6 background (Xu & Reichardt, unpublished observations). Heterozygous trkB knockout animals were separated from their wild-type littermates and housed in a conventional animal suite.

3.2.3: Animal surgery

All animal procedures were conducted in accordance with the Canadian guidelines for animal experimentation, and a local animal welfare committee. Under surgical metophane (methoxyfluorane) anaesthesia administered via nose cone, an incision was made on the right side at mid-thigh level to expose the right sciatic nerve. The tibial (TIB) and common peroneal (CP) branches of the sciatic nerve were dissected, separated 3-5 mm proximal to the natural trifucation, and freed from the surrounding connective tissue. As shown in Figure 3-1, the TIB nerve was cut distal to the popliteal artery and a 5 mm section was resected to prevent aberrant regeneration into the TIB distal nerve stump. The CP nerve was transected 3mm from its trifurcation from the TIB and sural branches, and the proximal stump was sutured to the innervated biceps femoris muscle to prevent regeneration (Figure 3-1B; Fu & Gordon, 1995). The proximal stump of the TIB nerve was sutured to the distal stump of the CP nerve within a 3 mm silastic tube with a single 8-0 suture (Figure 3-1C; Ethicon, Peterborough, ON). The skin and muscle were closed with 4-0 silk.

3.2.4: Evaluation of TIB motor axonal regeneration

As shown in Figure 3-1 D, intact TIB, and TIB motoneurons which regenerated their axons a distance of 10 mm from the site of initial injury and repair to the distal stump of the CP nerve were labeled with fluorogold and fluororuby, respectively, 2, 3, 4, 6, and 8 weeks after nerve repair. The details of the retrograde labeling procedure is described in Chapter 2.

3.2.4: Animal perfusion and tissue preparation

The mice were deeply anaesthetized with a ketamine/acepromazine cocktail (ketamine 150 mg/kg, acepromazine 5 mg/kg) for transcardial perfusion of 30 mL saline followed by 50 mL ice cold paraformaldehyde (4% paraformaldehyde in 0.1M phosphate buffer, pH=7.4). Solutions were administered at a rate of 5 mL/min using an infusion pump (Cole-Parmer Instrument Company, Model 74900). After perfusion, the T11-L1 spinal segments, which include the entire TIB motoneuron pool were removed (Swett et al., 1986). Following overnight post-fixation and cryoprotection in 4% paraformaldehyde, 30% sucrose, the cords were frozen in liquid nitrogen and stored at -70°C.

3.2.5: Enumeration of TIB motoneurons

As described in Chapter 2.

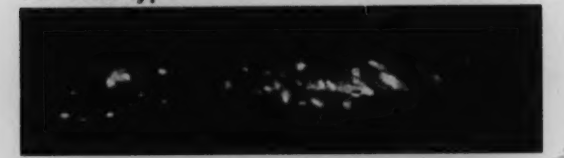
3.2.6: Statistics

Overall significant differences in the number of TIB motoneurons which regenerate their axons as a function of the time between the different strains of experimental mice were determined using a one-way ANOVA, and individual differences at each time point were evaluated using the Bonferroni method. Statistical significance was accepted at $p<0.05$.

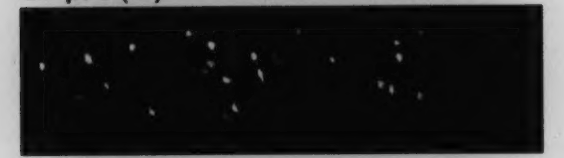
Figure 3-2: Reduced TIB motoneurons in p75 (-/-), but not trkB (+/-) mice compared to wild-type controls. The number of fluorogold labeled intact TIB motoneurons were counted in 50 μm longitudinal sections of the T11-L1 spinal segments. Compared to wild type controls (A), there was a significant reduction in the number of intact TIB motoneurons in p75 homozygous (-/-) knockout mice (B), but not in trkB heterozygous (+/-) knockout mice (C). *D.* Summary histogram of the number of fluorogold labeled intact TIB motoneurons.

Figure 3-2: Reduced number of intact TIB motoneurons in p75 (-/-) knockout mice compared to trkB (+/-) and wild type mice

A. Wild type

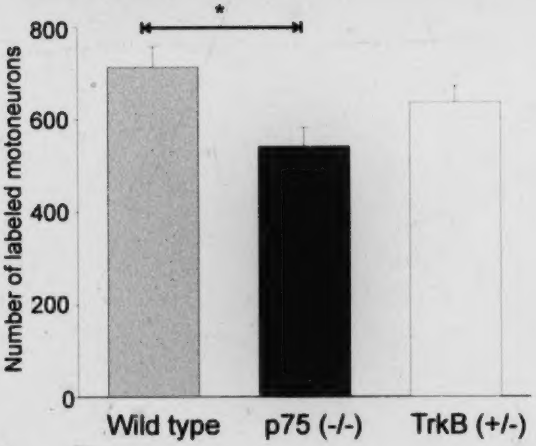


B. p75 (-/-)



C. TrkB (+/-)

D. Summary



3.3: Results

3.3.1: Reduced Number of TIB Motoneurons in p75 (-/-), but not trkB (+/-), Mice Compared to Wild-type Controls

There have been previous reports of reduced number of motoneurons in the facial motoneuron pool in both neonatal (Wiese et al., 1999) and adult (Ferri et al., 1998) p75 (-/-) mice compared to wild-type controls. To determine whether similar differences could be found in the TIB motoneuronal pool, the intact tibial nerve was labelled with fluorogold in both p75(-/-) mice and wild-type controls. The number of fluorogold labelled cells present in 50 μm longitudinal sections of the T11-L1 segments of the spinal cord were counted. As shown in representative sections in Figure 3-2A-C and summarised in Fig 3-2D, there was a significant reduction in the number of TIB motoneurons in p75 knockout mice compared to wild type controls, thus expanding the conclusions drawn by Wiese et al., (1999) that the p75 receptor may play a novel role in promoting motoneuronal survival during development, not only in brainstem motor nuclei, such as the facial nucleus, but in other motoneuronal pools as well.

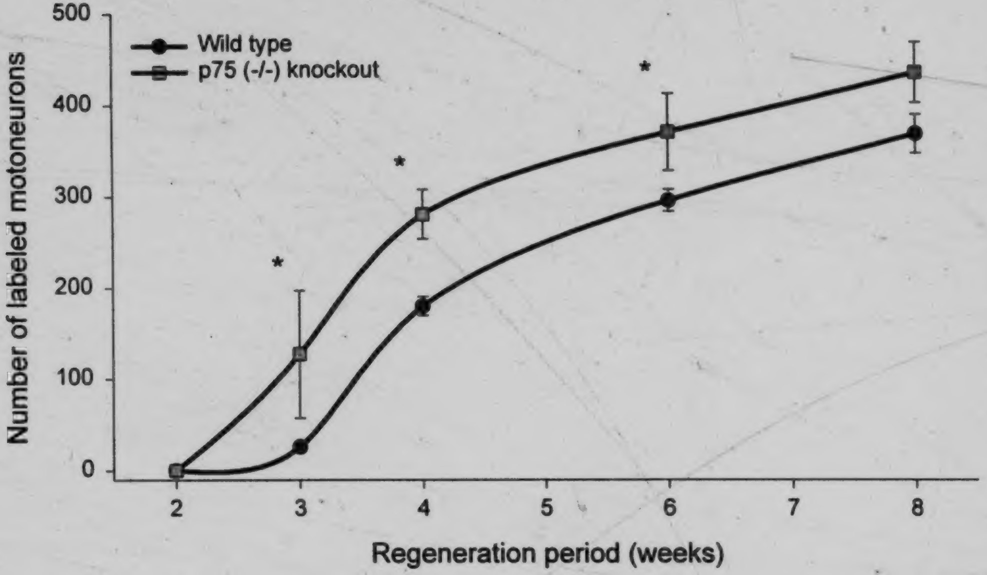
It was initially reported that mice carrying a homozygous deletion in the tyrosine kinase domain of trkB show significant loss of motoneurons, especially in the facial motoneuron pool (Klein et al., 1993). However, recent re-evaluation of these mice show no such deficits (Silos-Santiago et al., 1997; Alcantara et al., 1997). In addition to full length trkB receptors, motoneurons also express truncated isoforms of trkB (Barbacid, 1994). The role of these truncated receptors in development are currently unknown. To determine if both full length and truncated trkB receptors affect motoneuron survival into adulthood, we counted the number of fluorogold labelled intact TIB motoneurons in trkB (+/-) knockout mice as well as in wild-type controls. As shown in Figure 3-2, there is no significant difference in the number of TIB motoneurons between trkB (+/-) and wild type mice, suggesting that like full length trkB knockout mice, mice carrying a heterozygous deletion of both full length and truncated trkB receptors show no deficits in the number of motoneurons.

3.3.2: Increased motor axonal regeneration in p75 knockout mice compared to wild type controls

Motor axonal regeneration was evaluated by backlabelling TIB motoneurons which had regenerated their axons a distance of 10 mm from the site of TIB nerve section and cross-suture to the distal stump of the CP nerve at times of 2, 3, 4, 6, and 8 weeks later. Motoneurons which regenerated their axons were identified and quantified by labelling the CP nerve with fluororuby, and counting the number of fluororuby labelled TIB motoneurons in the T11-L1 spinal segments. As shown in Figure 3-3, at 2 weeks after nerve repair, there were no motoneurons which regenerated their axons in either wild-type or in the p75 (-/-) mice. However, one week later, at 3 weeks after nerve repair, there was significantly more motoneurons that regenerated axons in the p75 (-/-) mice compared to wild-type controls (Figure 3-3). This significant increase in motor axonal regeneration was detectable at 4 and 6 weeks post repair as well. At 8 weeks after nerve repair and axonal regeneration, the difference between the two strains of mice is no longer significant, suggesting that absence of the p75 receptor accelerates the outgrowth of motor axons across the suture site, but does not affect the total number of motoneurons which regenerate their axons by 8 weeks (Figure 3-3). However, in light of the significantly lower number of motoneurons in p75 (-/-) mice (Fig. 3-2), the relative number of motoneurons which regenerated their axons by 8 weeks (80%) was much higher in the p75(-/-) mice as compared to the wild type (50%). The progressive increase in the number of motoneurons which regenerate their axons over the 10 mm distance for both wild type and p75(-/-) mice is consistent with previous reports of delayed, or staggered regeneration of motor axons across the injury site (Al-Majed et al., 2000).

Figure 3-3: Increased TIB motor axonal regeneration in p75 (-/-) mice compared to wild type controls. The number of wild-type and p75 homozygous (-/-) knockout TIB motoneurons which regenerated axons a distance of 10 mm into the distal CP nerve stump and were labeled with fluororuby are presented as a function of increasing periods of regeneration after surgical repair to the distal CP nerve stump. There is significantly better motor axonal regeneration in the p75 (-/-) knockout mice compared to wild-type controls at 3, 4, and 6 weeks after nerve repair. By 8 weeks, the difference between the two groups of mice is no longer significant. *-denotes $p<0.05$.

Figure 3-3: Motor axonal regeneration is greater in p75 knockout mice compared to wild type controls



3.3.3: Motor axonal regeneration is initially accelerated in trkB (+/-) mice compared to wild type controls, but reaches an early plateau

Motor axonal regeneration was evaluated in trkB (+/-) mice 2, 3, 4, 6, and 8 weeks after TIB-CP cross suture. In surprising contrast to wild-type and p75 (-/-) mice which demonstrated no axonal regeneration until at least 3 weeks after nerve repair, in trkB (+/-) mice, there is substantial motor axonal regeneration after only 2 weeks of regeneration (Figure 3-4). This increased regenerative response is also apparent at 3 weeks (Figure 5 E), when there are 10 times as many TIB motoneurons which regenerate their axons in trkB (+/-) mice compared to wild-type controls (Figure 3-4). The number of motoneurons which regenerated their axons by 3 weeks in the trkB (+/-) mice constituted ~50% of the total number of contralateral intact TIB motoneurons; this number did not change significantly over the next 5 weeks (Figure 3-4). The failure of ~50% of the motoneurons to regenerate axons between 3 and 8 weeks after nerve repair differs from wild-type mice which show a progressive increase in the number of motoneurons which regenerate their axons across the injury site into the distal nerve stump (Figure 3-4). The reduced number of TIB motoneurons which regenerated their axons 8 weeks after nerve repair compared to wild-type and p75 (-/-) mice is evident in representative photomicrographs of the T11-L1 segments of the spinal cord (cf. Figure 3-5 B, D, F)

Figure 3-4: Initial acceleration of TIB motor axonal regeneration, in trkB heterozygous (+/-) knockout mice compared to wild-type controls, but reaches an early plateau. The number of wild-type and trkB heterozygous (+/-) knockout TIB motoneurons which regenerated axons a distance of 10 mm into the distal CP nerve stump and were labeled with fluororuby are presented as a function of increasing periods of regeneration after surgical repair to the distal CP nerve stump. Compared to wild type controls, there is significantly better motor axonal regeneration in the trkB (+/-) knockout mice compared to wild-type controls at early time periods (2 and 3 weeks) after nerve repair. However, at later time points (6 and 8 weeks after nerve repair), TIB motor axonal regeneration is significantly worse in trkB heterozygous knockout mice compared to wild-type controls. *-denotes $p<0.05$.

Figure 3-4: Motor axonal regeneration is initially accelerated in trkB (+/-) knockout micecompared to wild type controls, but reaches an early plateau

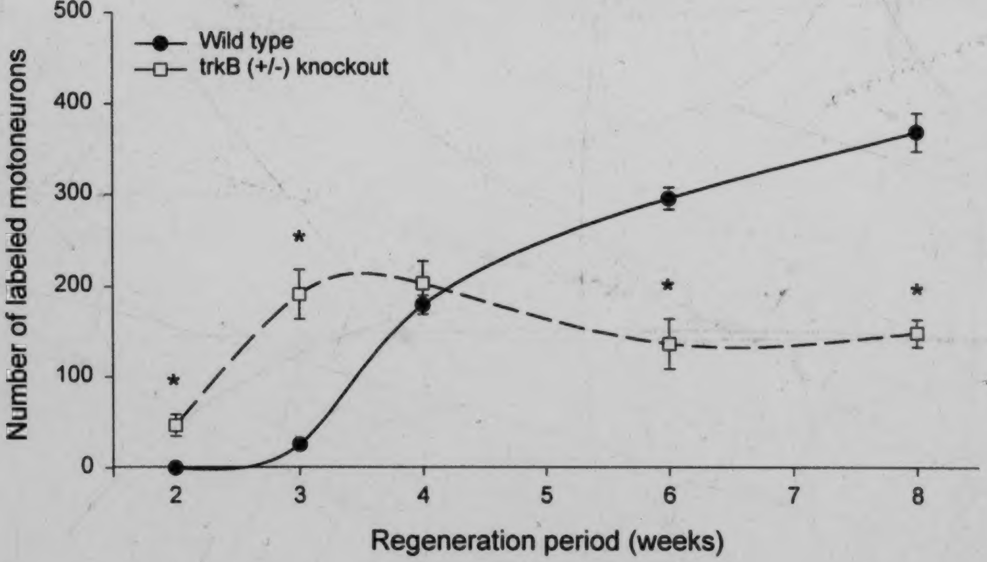
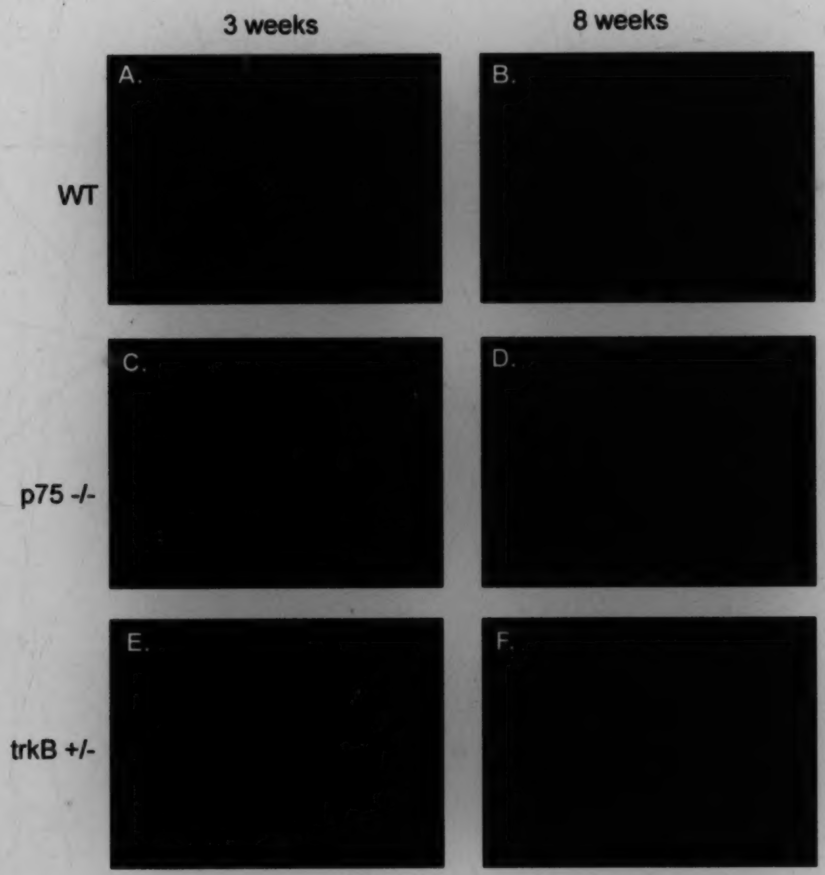


Figure 3-5: Deletions of p75 and trkB differentially affect the number of TIB motoneurons which regenerate their axons. Photomicrographs of fluororuby labelled motoneurons which regenerate their axons 10 mm from the site of nerve injury 3 weeks (*A,C,E*), and 8 (*B,D,F*) weeks after nerve repair. At 3 weeks, there is significantly greater TIB motor axonal regeneration in trkB (+/-) mice (*E*) compared to either p75 (-/-) mice (*C*), or wild-type control mice (*A*). In contrast, at 8 weeks, there is significantly greater motor axonal regeneration in p75 (-/-) mice (*D*), compared to wild type controls (*D*) or trkB (+/-) mice (*F*).

Figure 3-5: Fluororuby-labeled TIB motoneurons which regenerated axons after TIB-CP nerve repair in wild-type, p75 (-/-), and trkB (+/-) mice

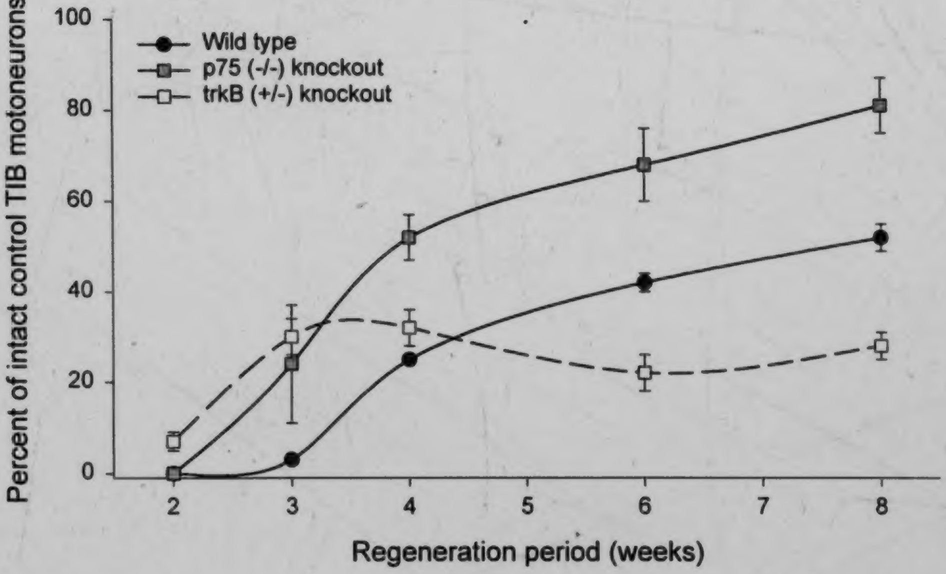

3 weeks
8 weeks
A.
B.
WT
C.
D.
p75 -/-
E.
F.
trkB +/-

3.3.4: Effects of p75 and trkB deletions are accentuated when normalized to contralateral intact controls

Compared with wild type controls, the increased axonal regeneration in p75 (-/-) mice at 3 and 8 weeks after TIB-CP cross-suture is evident in representative micrographs of fluororuby labeled motoneurons in 50 μm longitudinal sections of the T11-L1 segments of the spinal cord(Fig. 3-5). However, due to the significantly fewer intact TIB motoneurons in p75 (-/-) mice (Figure 3-2) a direct comparison between p75 (-/-) mice and wild type controls actually underestimates the accelerated motor axonal regeneration in p75(-/-) mice. Thus, as shown in Figure 3-6, when the number of motoneurons which regenerate their axons as a function of time is normalized to the number of intact TIB motoneurons for each individual strain, the effect of the p75 deletion of increasing the number of motoneurons which regenerate their axons is much more pronounced. The number of motoneurons which regenerate their axons a distance of 10 mm is significantly greater in p75 knockout mice at all time points examined. At the end of the study period (8 weeks), nearly all TIB motoneurons (~80%) had regenerated their axons in p75 (-/-) mice. This is in sharp contrast to the ~50% which regenerate in wild type controls (Figure 3-6). As there was no significant difference in the number of intact TIB motoneurons in trkB (+/-) mice compared to wild-type controls (Figure 3-2), normalizing the number of TIB motoneurons which regenerated their axons after nerve repair to the number of intact TIB motoneurons in trkB (+/-) mice did not affect the relative number of motoneurons which regenerated axons over time (Figure 3-6).

Figure 3-6: Motor axonal regeneration in wild-type, p75 (-/-), and trkB (+/-) mice normalized to the number of intact contralateral TIB motoneurons. Due to the reduced number of intact TIB motoneurons in p75 homozygous knockout mice compared to wild type controls; the increased motor axonal regeneration in p75 (-/-) mice is much more pronounced.

Figure 3-6: Motor axonal regeneration is differentially regulated by trkB and p75 receptors



3.4: DISCUSSION

To determine whether trkB and p75 differentially regulate motor axonal regeneration in response to endogenous neurotrophins, we have quantified motor axonal regeneration in mice that carry either a homozygous mutation in the p75 receptor (p75 -/-), or a heterozygous mutation in the transmembrane domain of the trkB receptor (trkB+/-). The results show that in addition to p75 being important for motoneuronal survival during development and into adulthood, its expression after injury may serve to inhibit motor axonal regeneration. In addition, although the expression of trkB receptors may not be critical for the early phase of motor axonal regeneration, full expression of trkB is critical for complete axonal regeneration to proceed.

3.4.1: Staggered motor axonal regeneration in wild type and p75 (-/-) knockout, but not trkB (+/-) knockout mice

The gradual increase in the number of motoneurons which regenerate their axons across the injury site and into the distal nerve stump has been recently described in a rat femoral nerve model of transection and repair (Al-Majed et al., 2000a). This staggered motor axonal regeneration is consistent with our findings of a progressive increase in the number of TIB motoneurons which regenerate axons 10 mm into the distal nerve stump as a function of time in p75 (-/-) knockout mice, as well as in wild-type controls (Figures 3-3, 3-6). However, the time course of motor axonal regeneration across the injury site and into the distal nerve stump is considerably prolonged in this study on mouse peripheral nerves. For example, by 8 to 10 weeks after nerve repair, all femoral motoneurons had regenerated their axons a distance of 25 mm in the rat (Al-Majed et al., 2000a), whereas at 8 weeks, ~80% and ~50% of p75 (-/-) and wild type TIB motoneurons, respectively, had regenerated their axons a distance of 10 mm in the mouse. These differences may be attributed to the particularly slow peripheral nerve regeneration observed following a nerve crush injury in C57Bl6-J mice (Lu et al., 1994), the wild type mice and background strain used in this study.

3.4.2: p75 as an inhibitor of axonal growth

There is an increasing accumulation of *in vitro* experimental evidence that the p75 receptor serves to inhibit the biological effects of neurotrophin signalling via their cognate trk receptors. In PC12 cells, activating p75 receptors reduced the ability of NGF to activate trkA (McPhee & Barker, 1997). In addition, if sympathetic neurons are maintained at sub-optimal concentrations of NGF, BDNF activation of p75 can antagonize trkA mediated survival (Aloyz et al., 1998; Bamji et al., 1998) and axonal growth (Kimpinski et al., 1997; Kohn et al., 1999). Moreover, preventing autocrine activation of p75 with a function blocking anti-p75 antibody directed towards the extracellular domain of p75, or with an anti-BDNF antibody also increased neurite outgrowth in these cells (Kohn et al., 1999). Furthermore, sympathetic neurons isolated from p75 knockout mice show increased neurite outgrowth, and do not show an inhibitory effect on axonal growth in response to BDNF compared to wild-type control neurons (Ibid).

In vivo experiments have also suggested a role for p75 in negatively regulating trk-mediated neurite growth. Sympathetic target organs such as the pineal gland which contain high levels of BDNF are hyperinnervated by tyrosine hydroxylase positive sympathetic axons in p75 knockout mice (Kohn et al., 1999). There is also extensive CNS sprouting of sympathetic and sensory fibres in p75 knockout mice which overexpress NGF under a glia-specific promoter (Walsh et al., 1999). This negative regulation of trk by p75 is not necessarily restricted to trkA, and may also extend to trkB, or trkC, as evidenced by increased motoneuronal survival and axonal sprouting following facial nerve crush in p75 knockout mice (Ferri et al., 1998).

Our results extend these findings to demonstrate that not only does the expression of p75 inhibit neurite outgrowth and axonal sprouting, but also reduces the number of motoneurons which regenerate their axons after injury, as well as the rate of axonal regeneration (Figure 3-3, 3-6). This is consistent with our previous findings that the inhibitory effects of high dose exogenous BDNF can be reversed by preventing BDNF binding to p75 receptors with function-blocking antibodies (Chapter 2). Thus, p75 mediates an inhibitory effect on motor axonal regeneration. The mechanism(s) by which p75 receptors mediate an inhibitory influence on axonal growth and regeneration *in vivo* are unclear. One possibility is that p75 signals independently to inhibit "growth/regeneration-promoting" signals occurring in neurons. One of the well documented downstream events following neurotrophin binding to p75 receptors is the activation of sphingomyelinases and induction of sphingomyelin hydrolysis, leading to the production of lipid second messenger ceramide (reviewed in Dobrowsky & Carter, 1998). Pharmacological elevations of intracellular ceramide, as well as exogenous application of short chain ceramide analogs has been shown to inhibit sympathetic neurite outgrowth *in vitro* (Posse de Chaves et al., 1997). Whether ceramide can inhibit motor axonal regeneration *in vivo* remains to be determined.

We cannot exclude the possibility that the increased motor axonal regeneration in p75 knockout mice is due to an indirect effect on the Schwann cells of the distal nerve stump. The p75 receptor is rapidly upregulated by Schwann cells after nerve injury (Heumann et al., 1987ab; Robertson et al., 1995; Taniuchi et al., 1988; Toma et al., 1992; You et al., 1996) and is associated with promoting migration (Anton et al., 1994) as well as apoptosis (Ferri & Bisby, 1999). The exact function of the non-neuronal expression of p75 remains to be determined.

3.4.3: trkB receptors differentially regulate motor axonal regeneration

It has been shown that expression of trkB receptors is critically important for preventing axotomy induced death in neonatal motoneurons (Alcantara et al., 1997). However, this is the first study to quantify the effects of reduced trkB expression on motor axonal regeneration. We have shown that motor axonal regeneration in trkB heterozygous knockout mice is significantly improved initially compared to wild-type controls during the early periods of regeneration. However, as time progresses, the number of motoneurons which regenerate their axons across the injury site and into the distal nerve stump fails to increase as occurs normally in wild type control mice (Figure 3-4, 3-6), and as previously described in the rat (Al-Majed et al., 2000).

The mutation in the transmembrane domain distinguishes the mice in this study from other trkB knockout mice previously used to evaluate the role of trkB receptors during development which carry a mutation in the tyrosine kinase domain, as deletion of the tyrosine kinase domain eliminates only full length trkB receptors (Klein et al., 1993; Silos-Santiago et al., 1997; Alcantara et al., 1997). In contrast, a mutation in the transmembrane domain eliminates both the full length as well as truncated trkB receptors (Xu & Reichardt, unpublished observations) which are expressed by motoneurons (Barbacid, 1994).

The explanation for the initial increase in motor axonal regeneration in the trkB (+/-) mice is likely linked to the non-neuronal expression of truncated trkB receptors. It was originally proposed that these truncated trkB receptors serve to present neurotrophins to regenerating axons in the distal nerve stump (Barbacid, 1994), as was suggested for the p75 receptor (Taniuchi et al., 1988). However, these neurotrophin receptors on the non-neuronal cells of the distal nerve stump may play a much more complex role in peripheral nerve regeneration. Recent evidence suggests that non-neuronal expression of truncated trkB receptors inhibits neurite growth. A substrate of fibroblasts stably expressing truncated trkB receptors actually inhibits neurite outgrowth by endocytosing bound neurotrophin and essentially removing trkB ligands from the environment of a growing neurite (Fryer et al., 1997). Thus expression of truncated trk receptors on the non-neuronal cells of the distal nerve stump may normally serve to restrict axonal growth and regeneration. Therefore, in the early stages of regeneration, prior to the delayed upregulation of BDNF in the Schwann cells of the distal nerve stump (Meyer et al., 1992; Figure 1-3), the reduced non-neuronal expression of truncated trkB receptors would have the effect of increasing the availability of the limited amounts of BDNF from the Schwann cells. The ~30% of TIB motoneurons which regenerate their axons in the early time points after nerve repair could be explained by this increased availability of BDNF for the reduced full length trkB receptors on motoneurons. That 30% does not increase further beyond 3 weeks despite the increasing synthesis of BDNF mRNA in Schwann cells to a maximum by 4 weeks (Figure 1-3, Meyer et al., 1992) because the reduced motoneuronal trkB expression is below threshold for responding to the available BDNF in the trkB (+/-) mice. Furthermore, reduced motoneuronal expression of trkB would alter the delicate balance between the positive effects of trkB, and the negative influence of p75 on motor axonal regeneration (Chapter 2) such that the inhibitory effects of p75 would dominate.

Our findings that exogenous BDNF completely reverses the negative effects of chronic axotomy on motor axonal regeneration (Chapter 2) is consistent with the failure of ~70% trkB (+/-) motoneurons to regenerate their axons at all over an 8 week period (Figure

3-4, 3-6). These findings emphasize the critical importance of BDNF and trkB receptors in sustaining motor axonal regeneration over extended periods of time as axons regenerate at the slow rate of 1-3 mm/day to reinnervate denervated muscle targets (*see* Sunderland, 1978).

3.4.4: Conclusions

In summary, this data shows that like neuronal survival after injury, motor axonal regeneration is depends on a delicate balance between trkB and p75 receptors. These results clearly demonstrate that the expression of p75 receptors serve to inhibit motor axonal regeneration, and that complete motor axonal regeneration depends on a full complement of trkB receptors.

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Chapter 4: The combined effects of GDNF and BDNF on motor axonal regeneration *in vivo*

4.1: Introduction

The poor functional recovery after peripheral nerve injury represents a surprisingly neglected clinical problem for which there is currently no effective treatment strategies. One of the major contributing factors to this clinical phenomenon is the progressive decline in the capacity of motoneurons to regenerate their axons during the prolonged period in which motoneurons remain axotomized prior to reinnervating denervated muscle targets (Fu & Gordon, 1995,1997). It has been suggested that neurotrophic factors that are expressed after nerve injury which are important for neuronal survival, may also be important for axonal regeneration (Meyer et al., 1992; Sendtner et al., 1992; Koliatsos et al., 1993; Kobayashi et al., 1996; Novikov et al., 1997; Naveilhan et al., 1997). However, neurotrophic factor expression after injury is generally transient (Chapter 1), and the failure to maintain the expression of neurotrophic factors over an extended period of time may explain, at least partially, the especially poor axonal regeneration of chronically axotomized motoneurons. Hence exogenous application of these neurotrophic factors may be able to sustain the regenerative capacity of chronically axotomized motoneurons.

We have previously described the effects of exogenous brain-derived neurotrophic factor (BDNF) on motor axonal regeneration after both immediate nerve repair, as well as nerve repair after chronic axotomy. BDNF belongs to the neurotrophin family of neurotrophic factors, which also includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). BDNF mediates its effects by binding two classes of receptors: a member of the tropomyosin receptor kinase (trk) family of receptors, trkB, and a member of the tumor necrosis family of receptors, p75 (Friedman & Greene, 1998; Yano & Chao, 2000). We have found that exogenous BDNF exerts a biphasic dose-dependent facilitation and inhibition of motor axonal regeneration (Chapter 2). The facilitatory and inhibitory effects of BDNF are attributed to BDNF binding to trkB and p75 receptors, respectively. This is consistent with decreased regeneration in trkB knockout mice, and increased regeneration in p75 knockout mice (Chapter 3).

Another candidate trophic factor for regenerating motoneurons is glial cell line-derived neurotrophic factor (GDNF). GDNF belongs to a subfamily within the TGF-β superfamily, together with neurturin, persephin, and artemin (Lin *et al.*, 1993; Kotzbauer *et al.*, 1996; Creedon *et al*, 1997). In contrast to BDNF, which binds to two distinct classes of receptors, GDNF mediates its effects via a single receptor complex. This receptor complex has two components, the ligand binding domain which is a glycosylphosphatidyl inositol membrane-linked receptor subunit called the GDNF-family receptor α-1 (GFRα-1), and a signal-transducing component, the tyrosine kinase, Ret (reviewed in Saarma & Sariola, 1999). The expression of GDNF in the denervated distal nerve stump and its receptors in axotomized motoneurons after injury are consistent with a functional role for GDNF in motor axonal regeneration. The receptors for GDNF, GFRα and Ret, are upregulated in axotomized motoneurons (Tsujino et al., 1999; Burazin et al., 1998; Naveilhan et al., 1997). GDNF is rapidly upregulated in denervated Schwann cells after sciatic nerve crush (Naveilhan et al., 1997), and is retrogradely transported by motoneurons (Yan et al., 1995).

There is considerable evidence demonstrating that GDNF is a potent survival factor for axotomized neonatal and adult motoneurons(Henderson et al., 1994; Yan et al., 1995; Oppenheim et al., 1995; Li et al., 1995; Vejsada et al., 1998; Yuan et al., 2000). In addition, overexpression of GDNF in developing muscle causes hyperinnervation of motor endplates during development (Nguyen et al., 1998) and adenoviral gene transfection of axotomized neonatal motoneurons with GDNF increases the numbers of myelinated axons in the distal nerve stump of the facial nerve after a crush injury, and this increased number of myelinated axons is associated with significantly improved whisker function compared to non transfected controls (Baumgartner & Shine, 1998).

In the present study, we will test whether exogenous GDNF, which does not bind p75 receptors, will mediate only positive effects on motor axonal regeneration under conditions of both acute and chronic axotomy.

4.2: Methods

4.2.1: Nerve repair and pump implantation

Chronic tibial (TIB) axotomy, the TIB-common peroneal (CP) nerve cross-suture, and pump implantation are shown in Figure 1 and were performed as described in Chapter 2.

4.2.2: Delivery of exogenous GDNF and BDNF via Alzet mini osmotic pumps

GDNF (kindly provided by Amgen Pharmaceuticals) was diluted in saline (0.9% NaCl) and administered via Alzet 28 day mini-osmotic pumps (model 2ML4) in doses of 0.1, 1 and 10 μg/day at a continual flow rate of 2.5 μl per hour. For delivery of combined BDNF and GDNF, the neurotrophic factors were diluted in saline to provide a daily dose of 0.1 μg/day GDNF, and 2 μg/day BDNF for a period of 28 days following delayed nerve repair after a 2 month period of chronic axotomy. To determine the duration of treatment required to promote regeneration of chronically axotomized motoneurons, combined BDNF and GDNF solutions were loaded into 7 day mini-osmotic pumps (Alzet model 2001) and the pumps were removed 1, 4, and 7 days after nerve repair. In control rats, the pumps were loaded with saline alone (n=10).

4.2.3: Retrograde labeling of TIB motoneurons

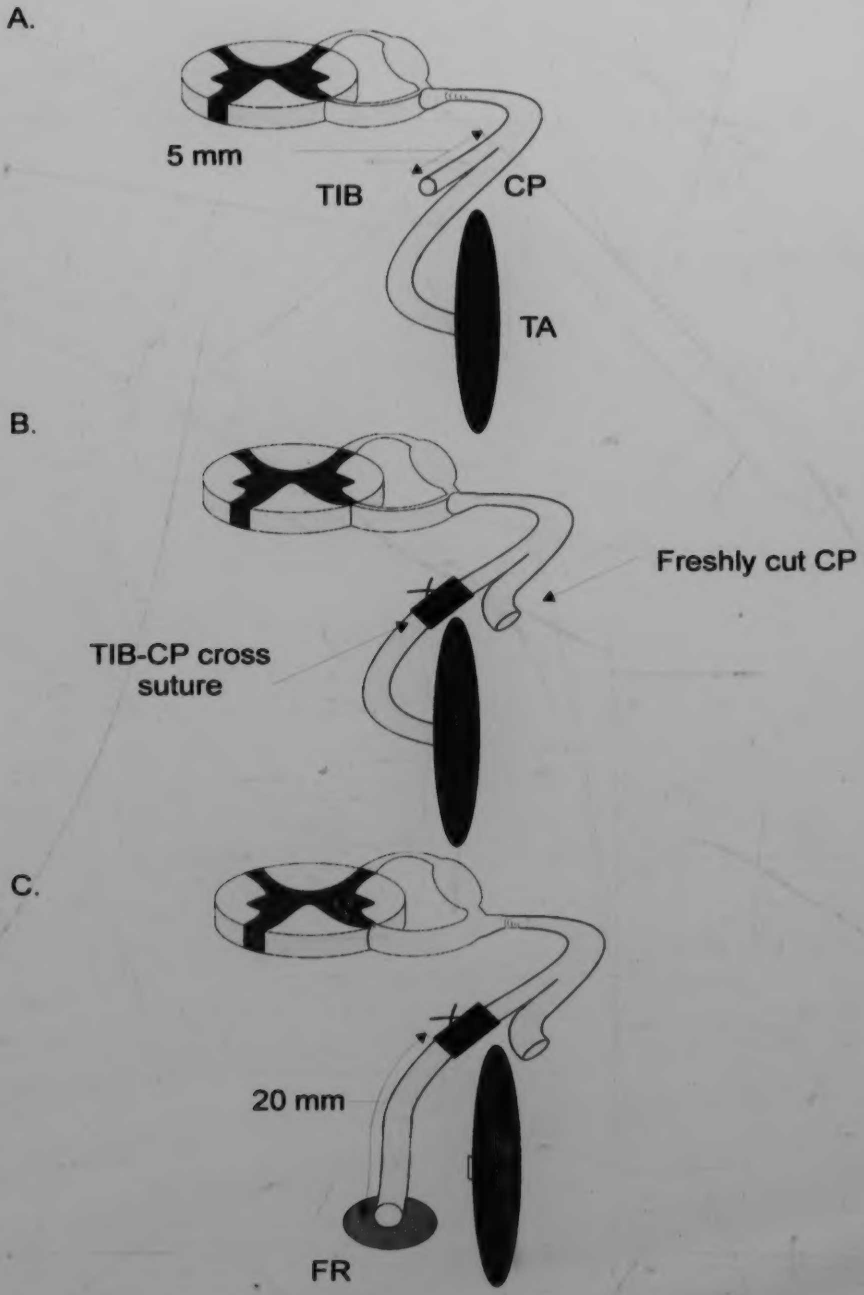
As described in Chapter 2.

4.2.4: Animal perfusion and tissue preparation

As described in Chapter 2.

Figure 4-1: Schematic representation of surgical procedures. *A*. The tibial (TIB) nerve was dissected and transected 5 mm distal to the trifurcation from the common peroneal (CP) and sural (not shown) branches of the sciatic nerve. For rats in which axonal regenerative capacity was reduced by chronic axotomy, the proximal TIB stump was sutured to surrounding innervated muscle to prevent regeneration for a 2 month period of chronic axotomy. *B*. Either immediately following transection or after a 2 month period of chronic axotomy, the proximal TIB nerve stump was cross-sutured to a freshly denervated CP distal nerve ump within a 5 mm silastic nerve cuff. Twenty-eight day mini-osmotic pumps were connected to the nerve cuff via a silastic catheter and implanted under the skin of the back. *C*. Axotomized TIB motoneurons which have regenerated their axons a distance of 20 mm are labeled with fluororuby (FR).

Figure 4-1: Schematic representation of surgical procedures


A.
5 mm
TIB
CP
TA
B.
Freshly cut CP
TIB-CP cross suture
C.
20 mm
FR

4.2.5: Enumeration of TIB motoneurons

As described in Chapter 2.

4.2.6: Preparation of nerves for electron microscopy

During the retrograde backlabelling surgery, a small (~3 mm) section of CP nerve distal to the site of dye application was dissected and removed prior to application of the dye. These nerve pieces were fixed in gluteraldehyde (3% in 0.1M phosphate buffer), stained with OsO_4 (3% solution in 0.1M phosphate buffer), dehydrated in ascending alcohols and embedded in araldite. Ultra thin sections were cut using a glass blade and examined using a scanning electron microscope. Black and white photographs were taken of representative sections through the distal nerve stump. A minimum of 3 animals were examined per group.

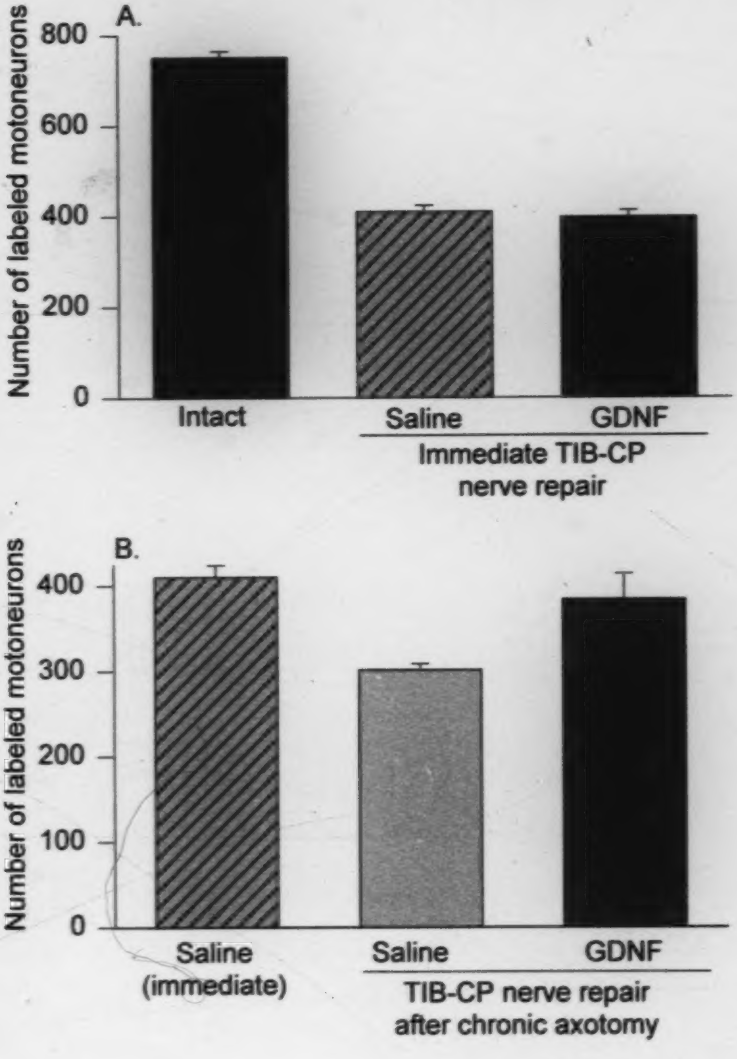
4.2.7: Statistics

Differences between groups were detected using independent T-tests. Statistical significance was accepted at the level of $p<0.05$. Values are presented as the mean ± SE.

To determine whether the combined effects of exogenous BDNF and GDNF had a significantly stronger effect on motor axonal regeneration than the sum of the effects of BDNF alone plus GDNF alone, the "bootstrap" method of random re-sampling (Efron, 1979) was used. This method has been previously described to evaluated the combined effects of BDNF and GDNF on motoneuronal survival (Vejsada et al., 1998). Briefly, prior to statistical analysis, the mean number of TIB motoneurons which regenerated their axons in saline control animals was subtracted from the number of TIB motoneurons which regenerated their axons in either the BDNF alone, GDNF alone, or combined BDNF and GDNF experimental groups. This subtraction was performed to remove the population of TIB motoneurons which would regenerate their axons in absence of any treatment and would thus be represented twice in the sum. The numbers were then divided into two groups, the mathematical sum of BDNF alone and GDNF alone (Group A), and the experimentally observed sum of combined BDNF and GDNF treatment (Group B). Fifty pairs consisting of n=5 values from group A and n=5 values from group B were constructed by random resampling the cell counts; repetition of individual values was allowed during this sampling procedure. Statistical significance between group A and group B was determined with the non-parametric Mann-Whitney U test (SPSS, version 10.0.7), the probability distribution of all 50 *P* values obtained was plotted, and the median and mean *P* values are reported.

Figure 4-2: Effects of exogenous GDNF on motor axonal regeneration after immediate nerve repair and nerve repair after chronic axotomy. *A*. Compared to saline controls (grey hatched bar, n=6), 0.1 µg/day exogenous GDNF (blue hatched bar, n=5) had no significant effect on the number of motoneurons which regenerated their axons after immediate nerve repair. The number of contralateral intact tibial motoneurons is shown for comparison (Chapter 2). *B*. Compared to immediate nerve repair (grey hatched bar, n=6 from panel A), a 2 month period of chronic axotomy significantly reduces the number of TIB motoneurons which regenerate their axons (grey bar, n=4). A dose of 0.1 µg/day exogenous GDNF (blue bar, n=4) completely reverses the negative effects of chronic axotomy.

Figure 4-2: Exogenous GDNF has no effect on motor axonal regeneration after immediate nerve repair, but completely reverses the negative effects of chronic axotomy



4.3: RESULTS

4.3.1: GDNF does not promote motor axonal regeneration after immediate nerve repair

As shown in Figure 4-2A, the mean (± SE) number of TIB motoneurons that regenerate their axons a distance of 20 mm into a freshly denervated CP distal nerve stump following immediate nerve repair and 28 days continuous saline infusion is 410 ± 28. This is significantly lower than the mean (±SE) number of motoneurons in the intact TIB motoneuron pool, 737 ±16 (Chapter 2). The ~55% of TIB motoneurons which regenerate their axons a distance of 20 mm in a 4 week period of time is consistent with our previous findings (Chapter 2), most likely due to previous findings that it takes at least twice as long (8-10 weeks) for all femoral motor axons to successfully cross the injury site and regenerate a comparable distance into a distal nerve stump (Al-Majed et al., 2000).

To examine the effects of exogenous GDNF on motor axonal regeneration, we counted the number of fluororuby labeled motoneurons which regenerated a distance of 20 mm into a freshly cut distal CP nerve stump in 4 weeks after immediate nerve repair. Continuous local application of exogenous GDNF for the 4 week period of axonal regeneration, at low doses of 0.1 μg/day which approximate the dose that was previously reported to promote sensory fiber regeneration after sciatic nerve injury in adult mice (Naveilhan et al., 1997), had no detectable effect on the number of TIB motoneurons which regenerated axons following immediate nerve repair as compared to the saline control (Fig. 4-2A). This is consistent with the findings of Chapter 2, in which exogenous BDNF had no beneficial effect on motor axonal regeneration after immediate nerve repair. Thus, in contrast to short periods of continuous electrical stimulation which increases the number of motor axons that cross the injury site into the distal nerve stump (Al-Majed et al., 2000), the neurotrophic factors GDNF and BDNF do not significantly accelerate regeneration.

The number of TIB motoneurons which regenerated their axons after a period of 2 month chronic axotomy prior to delayed nerve repair to a freshly denervated CP distal nerve stump was significantly reduced compared to immediate nerve repair (Fig. 4-2B). The low number of chronically axotomized motoneurons which regenerated their axons reflects a reduction to 75% of the regenerative capacity after immediate nerve repair. This reduction is comparable to the findings of a 66% reduction in the number of reinnervated motor units after the same 2 month period of chronic axotomy (Fu and Gordon, 1995). These data demonstrate that chronic axotomy reduces the capacity of TIB motoneurons to regenerate their axons, thereby extending the conclusions made by Fu and Gordon (1995) that chronic axotomy reduces the number of motor units which regenerate and reinnervate the denervated tibialis anterior muscle and confirming our previous findings (Chapter 2).

Continuous exogenous GDNF delivered to the site of nerve injury and repair, at a dose of 0.1 μg/day, significantly increased the number of 2 month chronically axotomized TIB motoneurons which regenerated their axons into a freshly denervated CP distal nerve stump compared to the saline controls (Figure 4-2B). The number of 2 month chronically axotomized TIB motoneurons which regenerated their axons after GDNF treatment was not significantly different from the number of motoneurons which regenerated their axons after immediate nerve repair. Thus, like low doses of exogneous BDNF, exogenous GDNF completely reversed the negative effects of chronic axotomy on motor axonal regeneration.

4.3.2: Exogenous GDNF does not show dose-dependent effects on motor axonal regeneration

To explore the possibility that the dose of GDNF (0.1 μg/day) was too low to increase motor axonal regeneration after immediate nerve repair, the dose was increased to 1 and 10 μg/day. As shown in Figure 4-3A, compared to saline control animals, exogenous GDNF was not able to promote TIB motor axonal regeneration after immediate nerve repair at any of the doses tested. Similarly, to explore possible dose-dependent effects of exogenous GDNF on motor axonal regeneration after a period of 2 month chronic axotomy, the range of doses tested was expanded to include 1 and 10 μg/day. Surprisingly, the initial dose of 0.1 μg./day appears to be saturating, as it is as effective at reversing the negative effects of chronic axotomy as 1 and 10 μg/day (Figure 4-3B). The lack of an observable dose-dependent effect on motor axonal regeneration for exogenous GDNF is in marked contrast the dramatic dose-dependent biphasic facilitatory and inhibitory effects of exogenous BDNF through a similar range of doses (Chapter 2).

Figure 4-3: GDNF shows only monophasic effects on motor axonal regeneration. *A.* Exogenous GDNF does not promote motor axonal regeneration after immediate nerve repair at any of the doses tested. *B.* In contrast to BDNF (Chapter 2), exogenous GDNF shows only facilitatory effects on motor axonal regeneration. Each data point represents the mean (±SE) of 4-5 animals per group.

Figure 4-3: Exogenous GDNF does not show dose-dependent effects on motor axonal regeneration

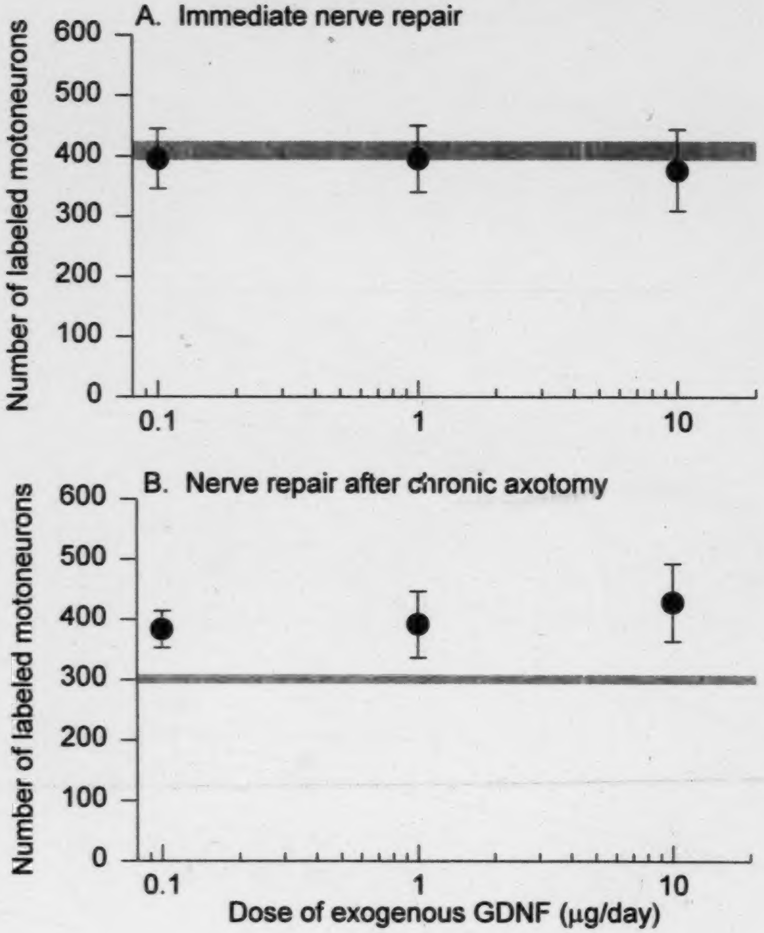
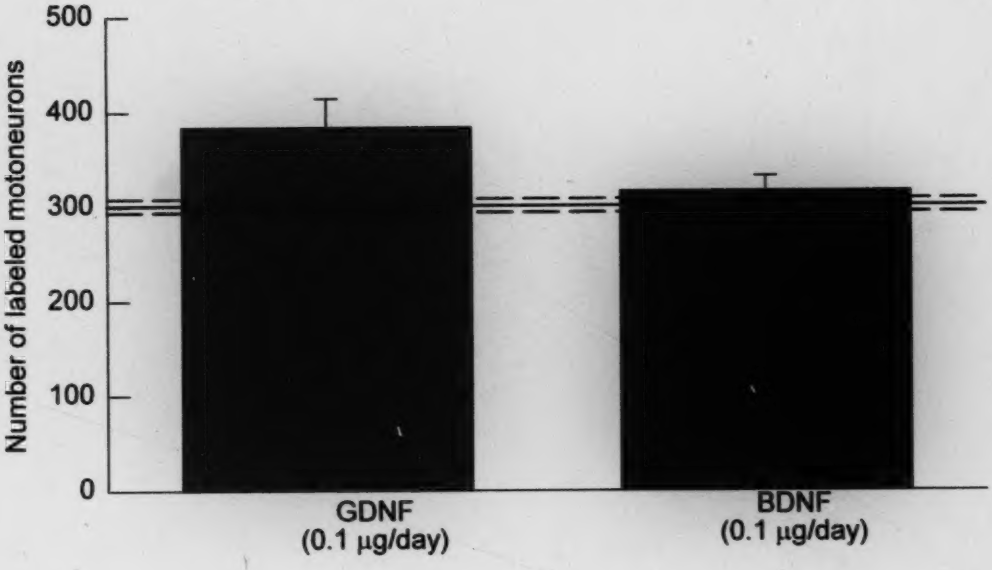


Figure 4-4: Comparison of relative potency of exogenous GDNF and BDNF. At the same dose of 0.1 μg/day, GDNF, but not BDNF significantly increases the number of chronically axotomized motoneurons which regenerated their axons.

Figure 4-4: GDNF is more potent than BDNF in promoting motor axonal regeneration after chronic axotomy



The potency of GDNF and BDNF in promoting motor axonal regeneration after chronic axotomy is compared in Figure 4-4. At the same low dose of 0.1 μg/day, exogenous GDNF is able to completely reverse the negative effects of chronic axotomy, but exogenous BDNF was without significant effect. However, as was demonstrated in Chapter 2, the substantially higher dose of 2 μg/day BDNF over the same 28 day period also completely reverses the negative effects of chronic axotomy. These results support the initial observations of Oppenheim et al. (1995) that GDNF is a much more potent neurotrophic factor than BDNF.

4.3.3: GDNF and BDNF show possible synergistic effects in promoting motor axonal regeneration

It has been previously reported that GDNF and BDNF can act together either synergistically in promoting the survival of axotomized neonatal motoneurons (Vejsada et al., 1998), or additively in promoting differentiation of embryonic motoneurons *in vitro* (Zurn et al., 1996). To determine the combined effects of GDNF and BDNF on TIB motor axonal regeneration *in vivo*, we administered the effective doses of 0.1 μg/day GDNF and 2 μg/day BDNF to 2 month chronically axotomized TIB motoneurons for 28 days. The combined GDNF and BDNF treatment substantially promoted axonal regeneration of chronically axotomized TIB motoneurons (Figure 4.5). This particularly strong effect of combined GDNF and BDNF treatment could possibly be due to synergistic interactions between the two neurotrophic factors, as this effect was significantly greater than the mathematical sum of beneficial effects of the two individual treatments (median of *P* distribution =0.032, mean of *P* distribution =0.048).

Figure 4-5: Possible synergistic effects of exogenous GDNF and BDNF on motor axonal regeneration. The number of chronically axotomized motoneurons which regenerated their axons in the presence of combined exogenous BDNF (2μg/day) and GDNF (0.1 μg/day) was significantly greater than the arithmetic sum of the beneficial effects of each individual facton (see text for details). Horizontal reference lines represent mean (±SE) number of motoneurons which regenerated axons in saline treated control animals. *-denotes significance compared to saline controls, **-denotes significance compared to individual neurotrophic factor treatments.

Figure 4-5: The combined effects of GDNF and BDNF are significantly greater than either neurotrophic factor alone

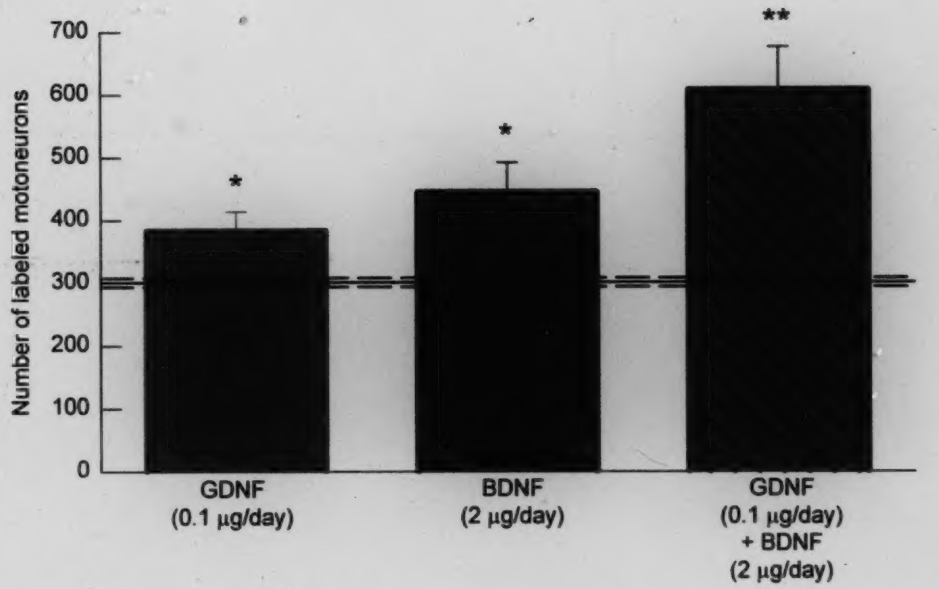
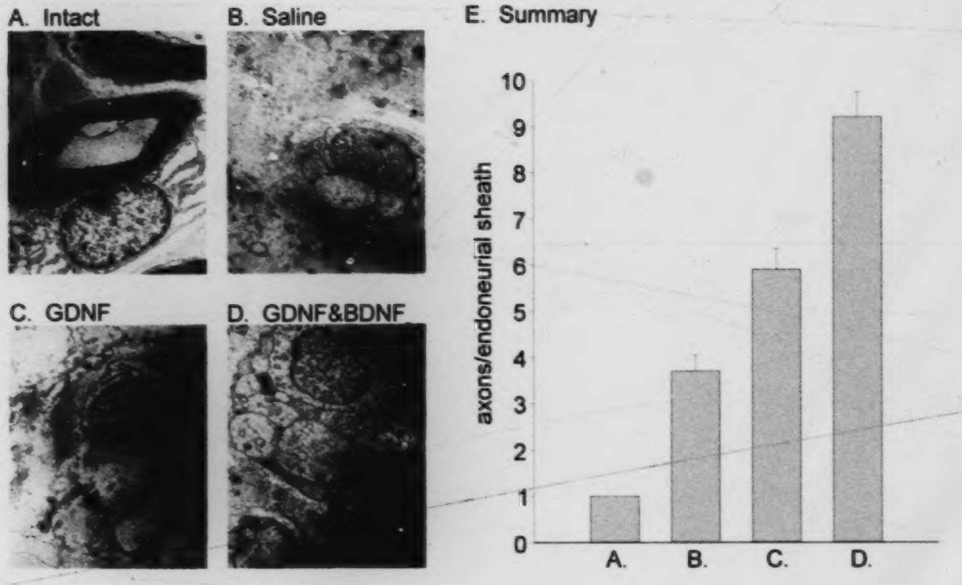


Figure 4-6: GDNF and BDNF promote regenerative sprouting after 28 days after peripheral nerve injury. *A-D.* Scanning electron micrographs of Schwann cells and their axons in an intact nerve (*A*), and a typical regenerating unit consisting of a Schwann cell and 4 daughter axons in saline controls (*B*). The number of axon sprouts/Schwann cell is increased following exogenous GDNF treatement (*C*), and dramatically increased following combined GDNF/BDNF treatment (*D*). (*E*) Summary histogram of the number of axons/endoneurial sheath for each treatment. Each bar represents the mean (±SE) of at least 3 animals per group in which 5 representative fields of the distal nerve stump were counted.

Figure 4-6: The ability of GDNF and BDNF to promote motor axonal regeneration is correlated with increased sprouting in the distal nerve stump


A. Intact
B. Saline
C. GDNF
D. GDNF&BDNF
E. Summary
axons/endoneurial sheath
0
1
2
3
4
5
6
7
8
9
10
A.
B.
C.
D.

4.3.4: BDNF and GDNF increase axonal sprouting

To determine whether the ability of GDNF, BDNF, and/or combined treatments to promote axonal regeneration of chronically axotomized motoneurons is correlated with morphological changes in the regenerating axons in the distal nerve stump, we removed a small portion of the nerve stump at the time of retrograde backlabelling of regenerated TIB motoneurons. Thus the segment includes only axons that have regenerated a distance of 20 mm from the site of initial nerve injury and repair. Tissue sections were evaluated using a scanning electron microscope, and representative sections are shown in Figure 4-6.

In intact nerves, a single Schwann cell is normally associated with only one axon (Figure 4-6A), however after nerve injury, a parent axon usually gives rise to 3-5 daughter axons and together they are called a regenerating unit (reviewed in Fu & Gordon, 1997). Schwann cells and their corresponding sprouting daughter axons in the regenerating unit are clearly visible in saline treated control animals, as shown in the representative scanning electron micrograph (Figure 4-6B). In GDNF treated animals, there is an increase in the number of axons that accompany a single Schwann cell (Figure 6C). Moreover, there is a profound and significant increase in the number of small regenerating axons accompanying a single Schwann cell in combined GDNF and BDNF treated animals (Figure 6E). The number of axonal sprouts per endoneurial sheath in combined GDNF and BDNF treated animals was significantly greater than GDNF alone (Figure 4-6D). Thus, there is a strong agreement between the ability of both GDNF alone, and in combination with BDNF, to promote motor axonal regeneration and increase the number of regenerating axons accompanying Schwann cells in the distal nerve stumps.

4.3.5: The beneficial effects of GDNF and BDNF are dependent on the duration of treatment

There have been conflicting reports with regards to whether chronic administrations of neurotrophic factors are necessary to elicit significant biological effects (Vejsada et al., 1998), or if similar long term biological effects can be elicited after a single dose (Kobayashi et al., 1997; Chai et al., 1999). To evaluate whether the substantial beneficial effects of combined GDNF and BDNF treatment on the axonal regeneration of chronically axotomized

TIB motoneurons are dependent on the duration of treatment, we performed the TIB-CP cross suture as described above for the long-term treated animals, but 7 day, instead of 28 day mini-osmotic pumps were used. These pumps were removed from their subcutaneous position 1, 4, and 7 days after nerve repair without trauma to the site of nerve injury and repair. As shown in Figure 4-7, there was no detectable effect of 1 or 4 days combined GDNF and BDNF treatment. A significant beneficial effect on motor axonal regeneration by exogenous application of GDNF and BDNF was detected only after 7 days treatment. However, the full 28 day combined GDNF and BDNF treatment was required for maximal beneficial effect. Thus in contrast to other studies which demonstrated that the biological effects of exogenous neurotrophic factors outlast the duration of treatment (Chai et al., 1999; Kobayashi et al., 1997), we demonstrated that continuous treatment with exogenous GDNF and BDNF is required for maximal benefit.

4.3.6: Continuous exogenous GDNF does not deter normal weight gain in experimental animals

There have been previous reports that high doses of exogenous GDNF can prevent normal weight gain in experimental animals (Vejsada et al., 1998; Giehl et al., 1997), thus we monitored the animals' weight throughout the experiments to determine whether continuous local delivery of exogenous GDNF to the site of nerve injury and repair, by osmotic pumps, would prevent normal weight gain in a dose dependent fashion. As shown in Figure 4-8A, in animals that underwent immediate TIB-CP cross suture, there was no significant difference in the weight gain by animals receiving either continuous saline, or GDNF at any of the doses tested. Furthermore, in animals that had the regenerative capacity of TIB motoneurons reduced by chronic axotomy for 2 months prior to cross suture to a freshly denervated CP distal nerve stump (Figure 4-9B), none of the GDNF doses tested significantly significantly affected of weight gained by the animals compared to the saline control animals. Although the percentage weight gain is less after onset of GDNF treatment for the animals in the chronic axotomy group compared to animals which underwent immediate nerve repair, this is more likely indicative of a plateau in normal growth, as saline control animals gained weight at a similarly lower rate.

Figure 4-7: Maximum beneficial effect of exogenous GDNF and BDNF requires sustained administration. Compared to saline controls (light grey horizontal reference line), a beneficial effect of combined GDNF and BDNF on the number of chronically axotomized motoneurons which regenerated their axons was apparent after 7 days, but not 1 or 3 days treatment. Each data point represents the mean (±SE) for 4-5 animals per group. For comparison, the number of intact TIB motoneurons is shown (dark grey horizontal reference line)

Figure 4-7: The ability of combined GDNF and BDNF to promote motor axonal regeneration of chronically axotomized TIB motoneurons depends on the duration of treatment

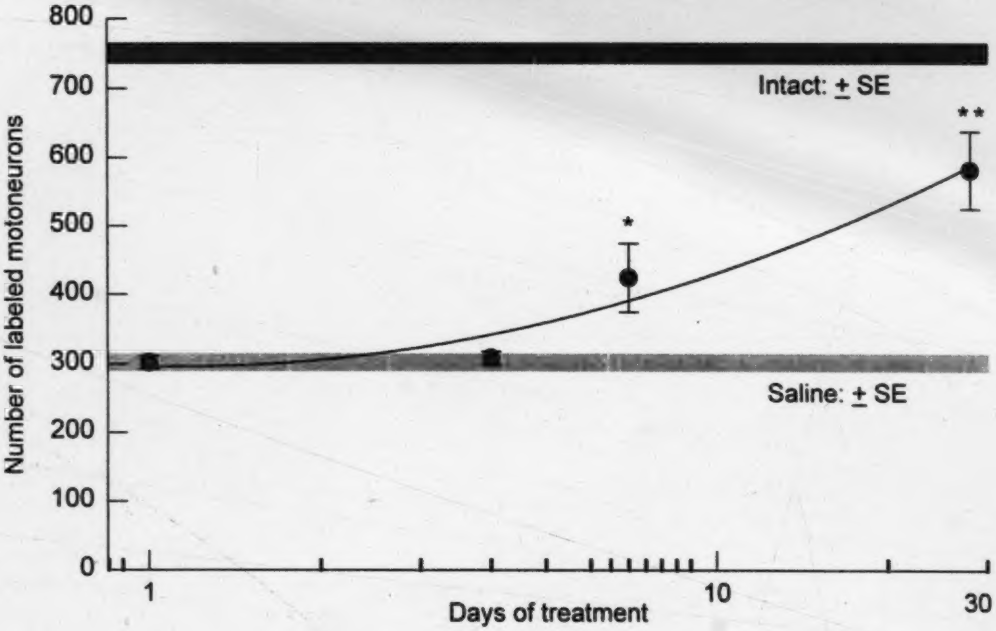
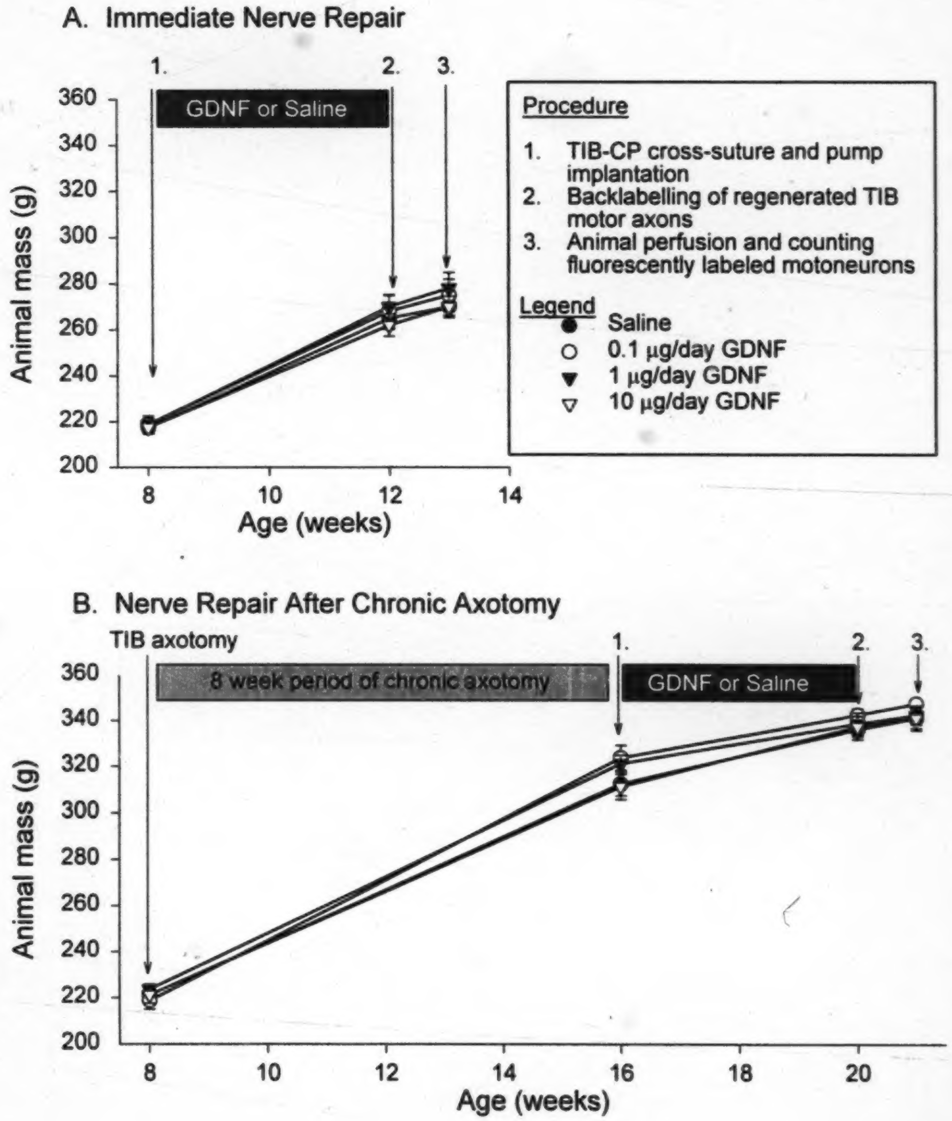


Figure 4-8: Exogenous GDNF does not reduce the amount of weight gain in experimental animals. Rats were weighed prior to the onset of each surgery. Animals in both immediate nerve repair groups (*A*), and nerve repair after chronic axotomy groups (*B*) showed no difference in the amount of weight gained regardless of treatment group.

Figure 4-8: Exogenous GDNF does not impede normal animal growth



4.4: DISCUSSION

This study uses a direct and quantitative assay to compare and contrast the effects of GDNF to BDNF in its ability to promote motor axonal regeneration, either alone or in combination. Similar to what was reported for BDNF, GDNF does not improve motor axonal regeneration after immediate nerve repair, suggesting that it does not accelerate regeneration across the injury site, yet GDNF completely reverses the negative effects of chronic axotomy on the number of motoneurons which regenerated their axons. However, in contrast to the effects reported in Chapter 2 for exogenous BDNF, GDNF does not show any inhibitory effects on motor axonal regeneration after either immediate nerve repair, or nerve repair after chronic axotomy. Furthermore, the combined effects of GDNF and BDNF on promoting motor axonal regeneration after chronic axotomy are possibly synergistic in nature, and this potent beneficial effect strongly depends on the duration of treatment. The beneficial effects of these GDNF and BDNF on motor axonal regeneration correlated well with their ability to increase axonal sprouting (Figure 4-6).

4.3.1: Exogenous neurotrophic factors sustain, not accelerate, motor axonal regeneration

In response to nerve injury, there are many changes which occur in both motoneurons, as well as in Schwann cells of the regenerative environment of the distal nerve stump, which has been denoted as a transition from a mature "transmitting" to a "regenerating" phenotype (Fu & Gordon, 1997). In particular, motoneurons downregulate enzymes associated with neurotransmission, such as ChAT (Yan et al., 1995; Friedman et al., 1995; Tuszynski et al., 1996; Kishino et al., 1997), and acetylcholinesterase (AChE; Fernandes et al., 1997; Kishino et al., 1997). In close temporal correlation with the decline in ChAT and AChE is an upregulation of regeneration associated genes, such as actin, GAP-43, and Tα1-tubulin (Miller et al., 1989; Tetzlaff et al., 1991), as well as neurotrophic factors and their receptors (reviewed in Fu & Gordon, 1997). In addition, denervated Schwann cells become a rich source of neurotrophic factors, such as BDNF and GDNF (Meyer et al., 1992; Funakoshi et al., 1993; Naveilhan et al., 1997; Trupp et al., 1997), and create a permissive growth environment for regenerating axons. Importantly however, upregulation of neurotrophic factor receptors in axotomized motoneurons is generally transient, and as time progresses, levels of neurotrophic factors and their receptors return to basal levels regardless of whether or not successful regeneration has occurred (Chapter 1, Figure 1-2). Specifically, although the functional receptors for BDNF (trkB and p75) and GDNF (GFRα-1 and Ret) are upregulated in motoneurons within 7 days of axotomy, this receptor mRNA returns to basal levels 6 weeks later (Hammarburg et al., 2000). Thus it appears that immediately after injury, motoneurons are at their peak regenerative capacity, but there is a time-limited and narrow window of opportunity for axonal regeneration to occur. It is possible that the immediate, but transient nature of the ability of motoneurons to successfully regenerate their axons may explain why exogenous neurotrophic factors, such as GDNF and BDNF, cannot accelerate motor axonal regeneration after immediate nerve repair but significantly improve the poor motor axonal regeneration observed after chronic axotomy (Chapter 2, Figure 4-2, 4-3). In other words, saturating levels of endogenous trophic support may be present after immediate nerve repair, but exogenous GDNF and BDNF may be required to sustain the regeneration of chronically axotomized motoneurons over extended periods of time.

4.3.2: GDNF and BDNF exert distinct effects on motor axonal regeneration

We have also demonstrated that *in vivo*, GDNF is much more potent than BDNF in promoting motor axonal regeneration after chronic axotomy (Figure 4-4). This is consistent with previous findings that GDNF is significantly more potent than BDNF in promoting motoneuronal survival, both of developing chick motoneurons (Oppenheim et al., 1995), and of neonatal motoneurons following sciatic nerve injury (Vejsada et al., 1998). Twenty times higher doses of BDNF were required to elicit the same positive effect in reversing the negative effects of chronic axotomy (Figure 4-4). In fact, the extremely low dose of 0.1 μg/day GDNF was a saturating dose, as there was no significant improvement in axonal regeneration at the higher doses of 1 or 10 μg/day (Figure 4-3). This monophasic dose-response curve for GDNF on motor axonal regeneration is in marked contrast to the biphasic dose-response effects demonstrated BDNF, where low doses (1-4 μg/day) promote, but higher doses (>4 μg/day) potently inhibit motor axonal regeneration (Figure 3-5). Since GDNF and BDNF belong to distinct families of neurotrophic factors, the lack of a biphasic dose-dependent response elicited by exogenous GDNF is consistent with the idea that the inhibitory effects of high dose BDNF are mediated by binding to p75 receptors (since GDNF does not bind to p75), and biphasic effects on motor axonal regeneration are not an inherent property that can be generalized to all neurotrophic factors

We have also provided evidence that GDNF and BDNF have synergistic positive effects on motor axonal regeneration after chronic axotomy (Figure 4-5). This is in agreement with the combination of a GDNF and BDNF supporting significantly more neonatal motoneurons after axotomy than either factor alone (Vejsada et al., 1998), and consistent with shared intracellular pathways induced by both GDNF and BDNF signaling. For example, both GDNF (Creedon et al., 1997; Hiwas et al., 1997; Wang et al., 1998) and BDNF (Ip and Yancopoulos, 1996; Segal and Greenberg; Kaplan and Miller, 1997) can activate Ras-MAP and PI3 kinase pathways. It has been recently demonstrated that GDNF and BDNF activated similar pathways to promote the survival of embryonic motoneurons (Sober et al., 1999; Dolce et al., 1999). At the cell membrane, GDNF binding to GFR-α1 and Ret induces tyrosine phosphorylation of Ret, and subsequent phosphorylation of MEK and Akt, indicative of MAP kinase and PI3 kinase activity, respectively (Sober et al., 1999). Likewise, BDNF binding to trkB also induces trkB phosphorylation, and downstream phosphorylation of MEK and Akt (Dolce et al., 1999). Pharmacological blockade of the PI3 kinase, but not the MAP kinase pathway prevented the survival promoting effects of both GDNF (Sober et al., 1999) and BDNF (Dolce et al., 1999). Thus there is both molecular and functional overlap of signaling pathways induced by both GDNF and BDNF stimulation which may explain the large beneficial effect of combined GDNF and BDNF treatment on promoting motor axonal regeneration.

4.3.3: Continuous exogenous neurotrophic factor treatment is required for maximal beneficial effect

There have been conflicting reports concerning the duration of neurotrophic factor treatment required to elicit long term effects in injured neurons. For instance, a single dose of BDNF was as effective as continuous treatment for the 6 week period following ventral root avulsion in maintaining motoneuronal survival (Chai et al., 1999). Similarly, the effect of BDNF in preventing axotomy-induced atrophy in rubrospinal neurons lasted up to 2 weeks following cessation of treatment (Kobayashi et al., 1997). In contrast, although a single dose of either BDNF or GDNF provided transient protection, continuous neurotrophic factor delivery was required to promote the long-term survival of axotomized neonatal motoneurons (Vejsada et al., 1998). Our findings are consistent with the latter study. We did not observe any significant benefit from the combined GDNF and BDNF treatment on the axonal regeneration of chronically axotomized motoneurons until after 7 days continuous treatment (Figure 4-7). Furthermore, maximum positive effect was elicited only when the combined treatment was given for the entire 28 day period of regeneration. One possible explanation for the delay in the ability of motoneurons to respond to exogenous application of neurotrophic factors is based on the fact that endocytosis and retrograde transport of GDNF and BDNF are receptor mediated (DiStephano et al., 1995; Oppenheim et al., 1995). Although the receptor mRNA required for GDNF and BDNF endocytosis and retrograde transport of GDNF and BDNF are upregulated early in axotomized motoneurons (<3 days), considerable delays may arise as a result of the long distance over which receptor proteins need to be anterogradely transported from the cell bodies in the ventral horn of the spinal cord to the site of nerve transection and exogenous application, or endogenous presence of neurotrophic factors. Thus, regenerating axons may not have the receptors present on their growth cones, and not be able to respond to local neurotrophic factor infusion, until several days after axotomy. This mechanism would resolve the apparent contradiction between the above studies concerning the duration of neurotrophin treatment. The experiments which demonstrated long lasting effects of neurotrophic factors after a single dose were applied in very close proximity to the cell bodies (Kobayashi et al., 1997; Chai et al., 1999), thus it might be assumed that the neurons would be able to respond almost immediately to exogenous neurotrophic factor application. However, in our experiments (Figure 4-8) and others (Vejsada et al., 1998) which have shown that continuous neurotrophic factor treatment is required to elicit beneficial effects, exogenous neurotrophic factors were applied peripherally to cut axons which may not be able to respond immediately to their local delivery.

4.3.4: Conclusions

In summary, these results demonstrate that the poor motor axonal regeneration observed after chronic axotomy can be reversed by exogenous application of continuous neurotrophic factors. Thus, it is possible that the poor functional recovery observed clinically as a result of traumatic peripheral nerve injury may be improved by promoting axonal regeneration of chronically axotomized motoneurons using long-term administration of low doses of neurotrophic factors, such as GDNF and BDNF, to sustain their regeneration over long periods of time.

4.5: References

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Chapter 5: Short chain ceramide analogs inhibit motor axonal regeneration *in vivo*

5.1: Introduction

The neurotrophins are a family of neurotrophic factors which are important mediators of neuronal survival and functional plasticity in the developing and adult central and peripheral nervous systems. In mammals, the neurotrophin family consists of nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5). Neurotrophins are unique among neurotrophic factors in that they mediate their effects via 2 classes of receptors: one of three members of the tropomyosin receptor kinase (trk) family of receptors, and a member of tumor-necrosis factor family of receptors p75 (reviewed in Yano & Chao, 2000). In contrast to p75 which binds all neurotrophins with similar affinity, the trk receptors are more ligand specific; NGF binds trkA, BDNF and NT-4/5 bind trkB, and NT-3 binds trkB and trkA, albeit to a lesser extent (Rodriguez-Tebar et al., 1992; Klein et al., 1991; Squinto et al., 1991, Lamballe et al., 1991).

Following peripheral nerve injury, axotomized motoneurons upregulate BDNF, trkB, and p75 receptors (Funakoshi et al., 1993; Kobayashi et al., 1996; Hammarburg et al., 2000). Using animals expressing a heterozygous deletion of trkB, or a homozygous deletion of p75, we have previously demonstrated that these receptors differentially regulate motor axonal regeneration. Expression of a full complement of trkB receptors is necessary for complete and successful motor axonal regeneration to occur, whereas the expression of p75 appears to inhibit or restrict axonal regeneration (Chapter 3). The inhibitory effects of the p75 receptor in motor axonal regeneration is consistent with several lines of *in vitro* and *in vivo* evidence that neurotrophin binding to p75 serves to inhibit trk-mediated signal transduction, neuronal survival, and axonal growth (Chapter 2; MacPhee & Barker, 1997; Aloyz et al., 1998; Kohn et al., 1999; Bamji et al., 1998; Ferri et al., 1998). However, the downstream mechanisms by which p75 mediates this inhibition remain unclear.

One of the well documented downstream events following neurotrophin binding to p75 receptors is the activation of sphingomyelinases and induction of sphingomyelin hydrolysis, leading to the production of ceramide (reviewed in Dobrowsky & Carter, 1998). Experiments which have demonstrated that elevations of intracellular ceramide and application of short chain ceramide analogs inhibits sympathetic neurite outgrowth *in vitro*

(Posse de Chaves et al., 1997) implicate ceramide as the intracellular mediator of the inhibitory effects of the p75 receptor *in vivo*.

In addition to the biological activities of ceramide, a second factor that may determine a cell's response to ceramide is the presence of enzymes involved in ceramide metabolism into biologically active metabolites, namely ceramidase and sphingosine kinase. Ceramidase cleaves ceramide into sphingosine and a free fatty acid, the sphingosine can be phosphorylated by sphingosine kinase to form the biologically active sphingosine-1-phosphate (SPP; Spiegel et al., 1996). SPP has been shown to reverse the pro-apoptotic functions of ceramide, thus suggesting that neuronal survival depends on a balance between ceramide and SPP (Cuvillier et al., 1996), and further, that regulation of the enzymes involved in ceramide metabolism may serve as important sites for regulation of cellular survival. For example, trkA activation via NGF induces a biphasic increase in sphingosine kinase activity in PC12 cells, and treatment with SPP protected these cells from apoptosis induced by serum withdrawal (Edsall et al., 1997).

The present study uses an *in vivo* model for the first time to quantify the effects of short chain ceramide analogs on the axonal regeneration of motoneurons which are either immediately repaired, or have had their regenerative potential reduced by chronic axotomy. In addition, we examine expression and regulation of sphingosine kinase in response to treatment with exogenous BDNF.

5.2: Methods

5.2.1: Nerve repair and pump implantation

Chronic tibial (TIB) axotomy, the TIB-common peroneal (CP) nerve cross-suture, and pump implantation were performed as described in Chapter 2.

5.2.2: Delivery of exogenous short chain ceramide analogs and BDNF via Alzet mini osmotic pumps

To evaluate the effects of ceramide on motor axonal regeneration after either immediate nerve repair (n=27) or nerve repair after chronic axotomy (n=15), short chain ceramide (C6-ceramide) and the biologically inactive isomer, C6 dihydroceramide (C6-DHC), were prepared from a 5 mM stock solution in dimethyl sulphoxide (DMSO) and diluted in saline (0.9 % NaCl) to a final concentration of 25 μM. The ceramide analogs were prepared in DMSO to facilitate permeability across cell membranes. C6-ceramide and C6-DHC were delivered via Alzet 28 day mini-osmotic pumps (model 2ML4; Alza Corp. Palo Alto, CA) at a continual flow rate of 2.5 μl per hour. In control rats, the pumps were loaded with DMSO (0.1%) in saline.

To determine whether sphingosine kinase activity is regulated by exogenous BDNF after either immediate nerve repair (n=12) or nerve repair after chronic axotomy (n=12), BDNF (kindly provided by Regeneron Pharmaceuticals) was diluted in saline and administered via Alzet 7 day mini-osmotic pumps (model 2001) in both low (2 μg/day) and high (20 μg/day) doses. In control animals, the pumps were loaded with saline alone.

5.2.3: Retrograde labeling of TIB motoneurons

As described in Chapter 2

5.2.4: Animal perfusion and tissue preparation

As described in Chapter 2

5.2.5: Evaluation of TIB motoneuronal survival

As described in Chapter 2

5.2.6: Enumeration of TIB motoneurons

As described in Chapter 2

5.2.7: Sphingosine kinase assay

Animals were deeply anaesthetized with sodium pentobarbitol (Somnitol; 50 mg/kg) one week after saline, low dose, or high dose BDNF treatment of TIB motoneurons after either immediate nerve repair, or nerve repair after a 2 month period of chronic axotomy, to the CP distal nerve stump. The TIB-CP nerve repair site was removed and dissected free from the silastic nerve cuff/drug delivery hardware and surrounding connective tissue. The tissue was washed several times in phosphate buffered saline (PBS; 0.1 M) and stored at -20°C in PBS until use.

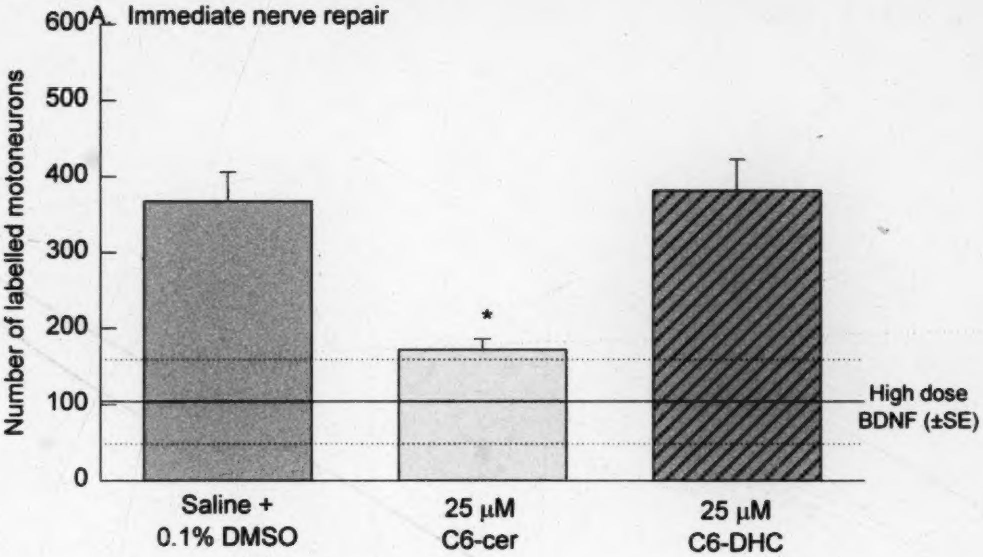
The isolated nerve samples were sonically homogenized in a sphingosine kinase buffer (prepared from a stock solution containing: 1 mL 1M Tris-HCl (pH=7.4), 200 μL 0.25M EDTA, 1.5 mL 0.5M NaF, 3.5 μL 2-mercapto ethanol, 500 μL Na_3PO_4, 50 μL of 10 mg/mL leupeptin/aproptinin, 50 μL of 10 mg/mL trypsin inhibitor, 432 mg β-glycerophosphate, 100 μL 0.2M PMSF, 20 mL 50% glycerol, and 26.1 mL distilled water). The cells were lysed by a series of freeze-thaw cycles between ice and water baths. After centrifugation, the protein pellet was reconstituted in the sphingosine kinase buffer solution, and the protein content was normalized across treatment groups to a total volume of 180 μL to which 10 μL of 1 mM sphingosine-BSA was added. Samples were incubated with the sphingosine substrate in the presence of 10 μL of 1 mM ATP-Mg^{2+} (containing ~10 μCi ^{32}P-ATP). The reaction mixture was incubated for 30 min at 37°C, and was stopped by adding 20 μL of 1 M HCl to each sample. After separating the incorporated ^{32}P from the unincorporated ^{32}P-ATP with a series of butanol-KCl washes, the amount of incorporated 32-P was evaluated by scintillation counting.

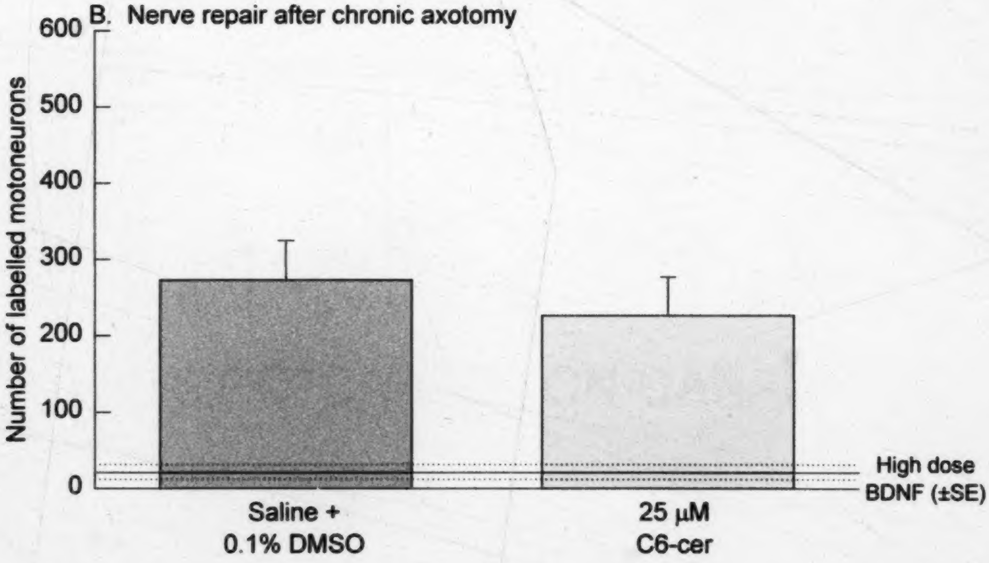
5.2.8: Statisitics

As described in Chapter 2.

Figure 5-1: Inhibitory effects of exogenous short chain ceramide analogs on the number of TIB motoneurons which regenerated their axons. *A*. Compared to saline + DMSO (0.1%) controls (grey bar, n=5), following immediate nerve TIB-CP cross-suture C6-ceramide (C6-cer; yellow bar, n=5) significantly inhibited motor axonal regeneration. The biologically inactive isomer, C6 dihydroceramide (C6-DHC; grey hatched bar, n=4) was without any detectable effect. *B*. Compared to saline + DMSO (0.1%) controls (grey bar, n=4), C6-cer was without any significant effect on the number of chronically axotomized TIB motoneurons which regenerated their axons into a freshly denervated distal nerve stump (yellow bar, n=4). Horizontal reference lines denote the reduced number of TIB motoneurons which regenerated their axons in the presence of high dose (20 μg/day) exogenous BDNF, shown for comparison (from Chapter 2).

Figure 5-1: Ceramide inhibits motor axonal regeneration after immediate nerve repair, but not after chronic axotomy





5.3: Results

5.3.1: Short chain ceramide analogs inhibit motor axonal regeneration after immediate nerve repair, but not nerve repair after chronic axotomy

It has been previously demonstrated that elevations of intracellular ceramide and application of short chain ceramide analogs inhibits sympathetic neurite outgrowth *in vitro* (Posse de Chaves et al., 1997). To determine whether the same short chain ceramide analogs inhibit motor axonal regeneration *in vivo*, we infused C6-cer via Alzet mini-osmotic pumps for the entire 28 day period of regeneration after either immediate nerve repair, or nerve repair after a 2 month period of chronic axotomy. As shown in Figure 5-1A, continuous infusion of saline (+ 0.1% DMSO) resulted in a mean number (±SE) of 368 ± 38 TIB motoneurons which regenerated their axons into the CP distal nerve stump after immediate nerve repair. This is not significantly different from the previously reported number of TIB motoneurons which regenerate their axons after immediate nerve repair in the presence of saline alone previously reported (Chapter 2, Chapter 4). Hence, DMSO has no detectable effect on motor axonal regeneration.

Continuous infusion of C6-ceramide (in saline + DMSO) significantly reduced the number of TIB motoneurons which regenerated their axons after immediate nerve repair to 171 ± 14 (Figure 5-1). This substantial inhibition of TIB motor axonal regeneration is comparable to the inhibitory effects observed following high dose exogenous BDNF previously reported (Chapter 2). Infusion of C6-DHC (in saline + DMSO), the biologically inactive isomer of C6-cer, did not have any significant effect on TIB motor axonal regeneration compared to saline controls. Therefore, the inhibitory effect of C6-ceramide is likely not due to any non-specific effects of long-term continuous infusion of short-chain lipids.

It has been previously demonstrated that exogenous high dose BDNF is significantly more potent in inhibiting the axonal regeneration of TIB motoneurons which have had their regenerative capacity reduced by chronic axotomy prior to repair to a freshly denervated CP distal nerve stump, as denoted as the horizontal reference lines in Figure 5-1 (Chapter 2). To determine whether C6-ceramide was also significantly more effective in inhibiting motor axonal regeneration after chronic axotomy than after immediate nerve repair, we infused either saline + DMSO or C6-cer to the site of the nerve repair site of a chronically axotomized TIB nerve to a freshly denervated CP distal nerve stump. As shown in Figure 5-1B, the mean (±SE) number of 2 month chronically axotomized TIB motoneurons which regenerated their axons into a freshly denervated CP distal nerve stump was 273 ± 51. This is significantly lower than the number of TIB motoneurons which regenerate their axons after immediate nerve repair, consistent with previous reports of reduced motor axonal regeneration following a 2 month period of chronic axotomy (Fu & Gordon, 1995; Chapter 2, Chapter 4). However, the number of TIB motoneurons which regenerate their axons in the presence of saline + DMSO is comparable to our previous reports motor axonal regeneration with saline alone (Chapter 2, Chapter 4). Thus, as with immediate nerve repair, axonal regeneration of chronically axotomized TIB motoneurons is not affected by the presence of DMSO.

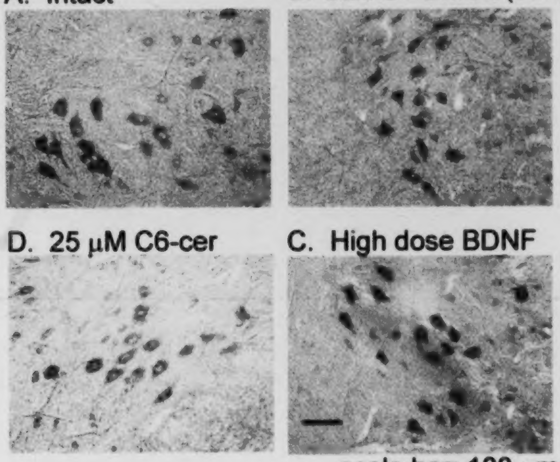
In contrast to the potent inhibitory effect of C6-ceramide on TIB motor axonal regeneration after immediate nerve repair, exogenous application of C6-ceramide was without any detectable effect on TIB motor axonal regeneration after chronic axotomy compared to saline + DMSO controls (Figure 5-1B). As there was no significant observable effect with continuous infusion of C6-cer, controls with C6-DHC were not performed.

5.3.2: C6-ceramide is not neurotoxic

Intracellular generation of ceramide is an event that is intimately linked to the pro-apoptotic effects of neurotrophins binding to p75, as well as other TNF-α family members binding to their cognate receptors (reviewed in Perry & Hannun, 1998; Chao et al., 1998; Barker et al., 1998; Barrett, 2000). Therefore, we explored the possibility that reduced number of TIB motoneurons which regenerated their axons after immediate nerve repair was due to the long-term application of short chain ceramide analogs causing cell death. In a separate group of animals, we counted the number of Nissl-positive motoneurons in every 5th 40 μm section through the T11-L1 spinal segments, which includes the entire TIB motoneuronal pool (Swett et al., 1986). As shown in Figure 5-2, compared to the number of motoneurons in the contralateral motoneuronal pool (Figure 5-2A), there was no difference in the number of motoneurons which have been treated with saline + DMSO (Figure 5-2 B), or C6-ceramide (Figure 5-2C). The number of motoneurons in the T11-L1 spinal segments following long-term continuous BDNF treatment is shown for comparison (Figure 5-2D). Adjacent sections stained with Hoescht nuclear stain and TUNEL did not show any apoptotic nuclei (data not shown).

Figure 5-2: Short chain ceramide analogs are not neurotoxic to axotomized motoneurons. *A-D*. Representative photomicrographs of Nissl-positive motoneurons in the T11-L1 spinal segments on the intact contralateral side (*A*), or following saline + DMSO (*B*), C6-ceramide (C6-cer), or (*D*) high dose (20 µg/day; from Chapter 2) exogenous BDNF treatment. *E*. The number of Nissl-positive motoneurons were counted in every 4th 40 µm cross section of the T11-L1 spinal segments of the spinal cord. Compared to the number of Nissl-positive motoneurons in the intact contralateral side (black bar), there was no significant difference in the number of Nissl-positive motoneurons following saline + DMSO (grey bar), C6-cer (yellow bar), or high dose BDNF (from Chapter 2, red bar) treatment.

Figure 5-2: High dose BDNF and C6 ceramide do not reduce the number of motoneurons in the TIB motoneuron pool



A. Intact

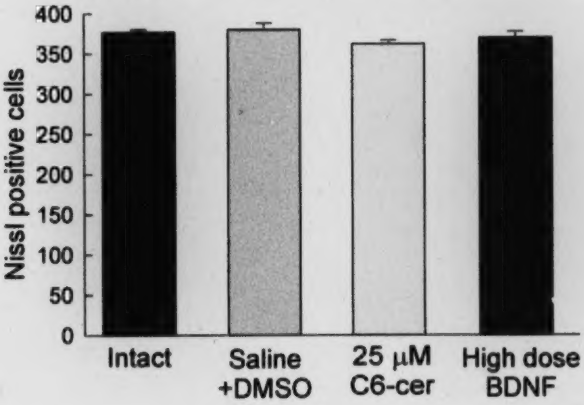
B. Saline+ DMSO(0.1%)

D. 25 μM C6-cer

C. High dose BDNF

scale bar: 100 μm

E. Summary



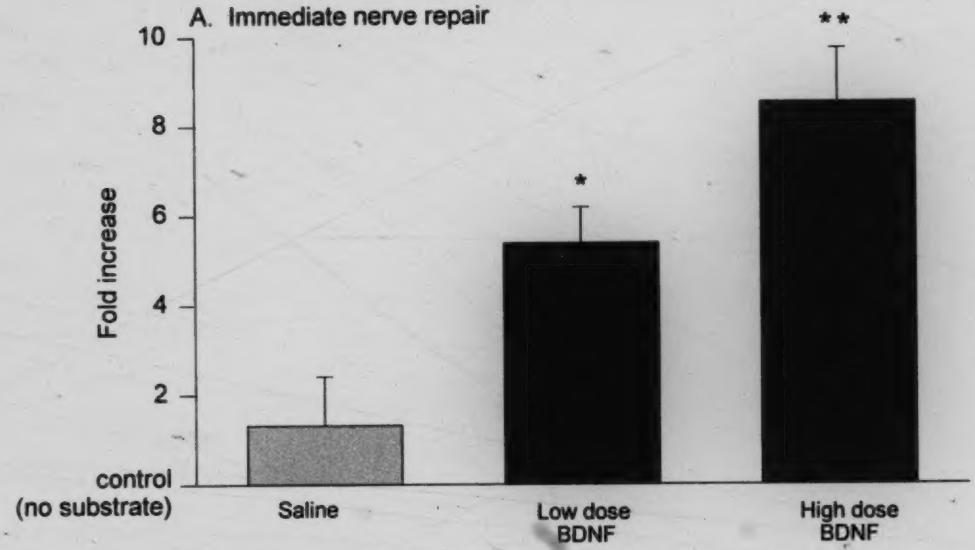
5.3.4: Exogenous BDNF induces sphingosine kinase activity after immediate nerve repair, but not nerve repair after chronic axotomy

We have previously demonstrated that exogenous application of high dose BDNF inhibits motor axonal regeneration both after immediate nerve repair as well as nerve repair after chronic axotomy, the effects being much more potent in the latter case (*see* Figure 5-1; Chapter 2). However, as described above, exogenous application of short chain ceramide analogs only inhibits motor axonal regeneration after immediate nerve repair. This suggests that ceramide may be involved in mediating the inhibitory effects of high dose BDNF after immediate nerve repair, but different mechanisms are involved in mediating the inhibitory effects of high dose BDNF after chronic axotomy. Perry et al (1998) suggest that one of the key determinants of a cell's response to ceramide is the activities of ceramide metabolizing enzymes. As it has been previously demonstrated that NGF induces sphingosine kinase activity in PC12 cells *in vitro* (Edsall et al., 1997), we tested the possibility that the differential effectiveness of ceramide on motor axonal regeneration after immediate versus delayed nerve repair after chronic axotomy is due to a differential regulation of the enzyme sphingosine kinase in the conversion of sphingosine to SPP. The TIB-CP nerve repair site was removed after 7 days treatment with saline, low dose (2 μg/day), or high dose (20 μg/day) exogenous BDNF.

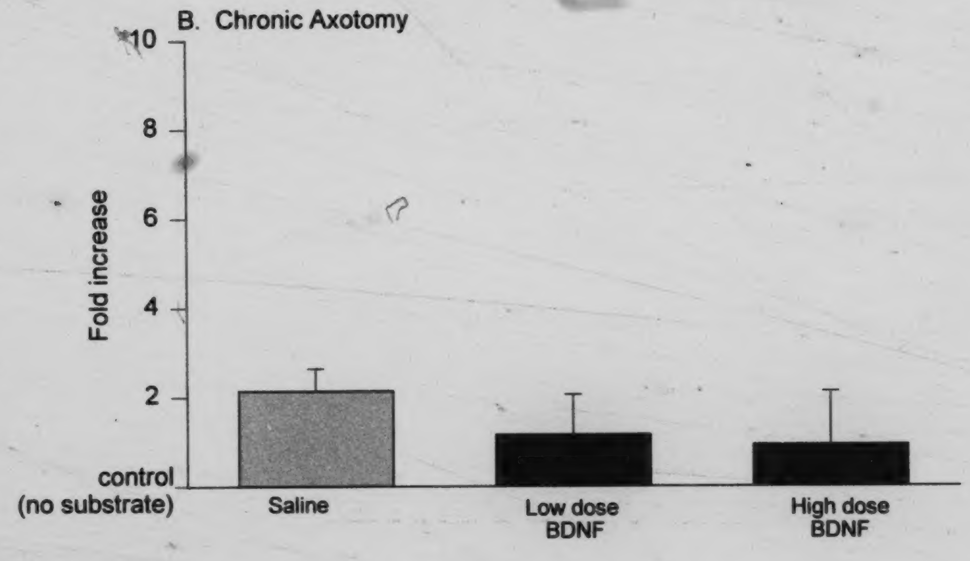
Sphingosine kinase activity was assayed by scintillation counting of ^{32}P incorporation following sample incubation with a sphingosine substrate. As shown in Figure 5-3, there is a significant BDNF dose-dependent upregulation of sphingosine kinase activity 7 days following immediate nerve repair (Figure 5-3A). Low dose BDNF induced a mean (±SE) 5.4 ± 1.15 fold increase in sphingosine kinase activity, and high dose induced a significantly higher 8.5 ± 0.9 fold increase. However, no induction of sphingosine kinase activity was detected after chronic axotomy (Figure 5-3B). Therefore, consistent with the effects of exogenous short chain ceramide analogs (Figure 5-1), a strong role for ceramide and/or its metabolites are implicated in motor axonal regeneration after immediate nerve repair, but not after chronic axotomy.

Figure 5-3: Exogenous BDNF induces sphingosine kinase activity. Sphingosine kinase activity was assayed by scintillation counting of ^{32}P incorporation following incubation with a sphingosine substrate. Tissue was analyzed 7 days following saline (grey bars, n=7), low dose BDNF (2 µg/day; blue bars, n=7), or high dose BDNF (20 µg/day; red bars, n=6). Following immediate nerve repair (*A*), exogenous BDNF induced a significant dose-dependent increase in sphingosine kinase activity, whereas no increase was detectable after chronic axotomy (*B*). * denotes significantly greater than saline controls, ** denotes significance compared to low dose BDNF.

Figure 5-3: Exogenous BDNF induces sphingosine kinase activity in a dose-dependent fashion after immediate nerve repair, but not after chronic axotomy



A. Immediate nerve repair



B. Chronic Axotomy

5.4: Discussion

The present study is the first to evaluate the role of ceramide and ceramide metabolism *in vivo*, specifically in motor axonal regeneration in conditions of acute and chronic motoneuronal axotomy. We demonstrate that short chain (C6) ceramide analogs mimic the effects of high dose exogenous BDNF by being potent inhibitors of motor axonal regeneration after immediate nerve repair, but not after chronic axotomy. A role for ceramide and/or its metabolites in mediating this inhibitory effect only after immediate nerve repair is supported by the ability of exogenous BDNF to increase activity of sphingosine kinase, an enzyme involved in ceramide metabolism, only after immediate nerve repair.

There are several possible mechanistic explanations for the inhibitory effect of ceramide on motor axonal regeneration. First, it is possible that ceramide reduces the ability of trkB to respond to exogenous BDNF. This is supported by *in vitro* evidence that short term application of short chain ceramide analogs reduce trkA phosphorylation in response to NGF (MacPhee & Barker, 1997). However, long term application of the same short chain ceramide analogs actually increase trkA phosphorylation in response to NGF (MacPhee & Barker, 1999). Thus, although it is clear that ceramide modulates trkA activation *in vitro*, the nature of this regulation and whether or not it translates to other members of the trk family of receptors remains to be elucidated *in vivo*.

The ability of exogenous BDNF to induce sphingosine kinase activity *in vivo* implicates ceramide metabolites in mediating its effects on motor axonal regeneration. Specifically, an increase in sphingosine kinase activity suggests an increase in the production of SPP. In contrast to the p75-mediated generation of ceramide, the induction of sphingosine kinase activity may be a trkB mediated event. This is consistent with experiments which demonstrate that the NGF-mediated induction of sphingosine kinase activity in PC12 cells *in vitro* is a trkA-mediated effect, as blocking tyrosine kinase activity with K252a eliminated the ability of NGF to increase sphingosine kinase activity (Edsall et al., 1997). SPP has been demonstrated to act both as an intracellular signalling molecule, in which it serves to counteract several ceramide-mediated effects, and SPP can also mediate ligand specific effects via binding to the EDG-1 family of G-protein coupled receptors (reviewed in Pyne

& Pyne, 2000; Spiegel et al., 1998; Spiegel, 1999; 2000). SPP has been recently demonstrated to cause neurite retraction in PC12 cells via a ligand-dependent manner (Sato et al., 1997). Thus it is possible that the BDNF induced increase in sphingosine kinase activity acts to increase SPP levels, which inhibit motor axonal regeneration by binding to EDG receptors on axotomized motoneurons. The exact mechanisms by which SPP affects motor axonal regeneration *in vivo* remains to be determined.

An alternative mechanism of action for ceramide in motor axonal regeneration is via inhibition of downstream "growth-promoting" signals in regenerating motoneurons. In light of a dramatic reduction in the number of motoneurons which regenerate their axons in heterozygous trkB knockout mice (Chapter 3), the beneficial effects of low doses of exogenous BDNF (Chapter 2) are attributed to the trkB receptors expressed on axotomized motoneurons. Intracellular signals activated in response to BDNF binding to trkB receptors include the PI3K-Akt pathway and the ras-erk pathway (*see* Chapter 1; Dolcet et al., 1999; Atwal et al., 2000). In axotomized motoneurons, the PI3K-Akt pathway has been demonstrated to be an important mediator of both survival and axonal regeneration (Namikawa et al., 2000). Ceramide has been demonstrated to dephosphorylate Akt, thus inactivating the PI3K/Akt pathway (Kim et al., 2001, Zinda et al., 2001, Schubert et al., 2000), thus providing a possible downstream mechanism by which the p75-mediated generation of ceramide can inhibit a trkB-mediated growth promoting signal.

Although it has been shown that ceramide can mediate intracellular events in Schwann cells *in vitro*, such as induction of NF-κB (Carter et al., 1996), it is unlikely that the effects of ceramide on motor axonal regeneration are mediated by an indirect effect on the Schwann cells of the distal nerve stump. An indirect effect on Schwann cells would be detectable in conditions of both acute and chronic axotomy, as motor axons are regenerating into a freshly denervated distal nerve stump in both conditions. In contrast, ceramide inhibits motor axonal regeneration after only immediate nerve repair (Figure 5-1) strongly suggesting that the effects of ceramide are mediated in axotomized motoneurons, not the Schwann cells of the distal nerve stump.

Despite the clear involvement of ceramide and/or its metabolites in axonal regeneration following immediate nerve repair, our data suggest that alternative mechanisms are involved in the axonal regeneration of chronically axotomized motoneurons. We have previously demonstrated that preventing BDNF binding to p75 receptors with a function-blocking antibody reverses the inhibitory effects of high dose BDNF after both immediate nerve repair, and chronic axotomy (Chapter 2). This suggests that events other than the generation of ceramide downstream of BDNF binding to p75 receptors are involved in mediating the inhibitory effects of high dose BDNF on the axonal regeneration of chronically axotomized motoneurons. Recent *in vitro* experiments suggest that neurotrophin binding to p75 may activate pathways distinct from the induction of sphingomyelinase activity. Other members of the TNF family of receptors, to which p75 belongs, interact with TNF receptor associated factors (TRAFs) via a conserved intracellular element termed the "death domain", to modulate apoptosis, as well as JNK and NF-κB activity (Rothe et al., 1995; Song et al., 1997). The cytoplasmic juxtamembrane region tail of p75 has been shown to associate with TRAF6 in a ligand-dependent manner (Khursigara et al., 1999). In the same study, a dominant negative form of TRAF6 was shown to inhibit the p75-dependent NF-κB nuclear translocation in response to NGF treatment. Furthermore, several other adaptor proteins have been shown to interact with the p75 receptor in GST pulldown assays, such as neurotrophin receptor interacting factor (NRIF), SC-1, and neurotrophin receptor-interacting melanoma antigen gene homolog (NRAGE) (reviewed in Kaplan & Miller, 2000). These adaptor proteins are associated with apoptosis (NRIF) and cell cycle arrest (SC-1 and NRAGE), but their role in axonal growth or regeneration remains undetermined. Whether or not these other pathways are present in axotomized motoneurons, and their role in mediating the effects of exogenous neurotrophic factors *in vivo* remain to be determined.

In summary, these experiments are the first to demonstrate that ceramide inhibits motor axonal regeneration *in vivo*, and that sphingosine kinase activity can be regulated by exogenous BDNF. The regulation of motor axonal regeneration by exogenous neurotrophic factors is likely much more complex than originally believed, and the role of ceramide and its metabolites in the motoneuronal response to injury clearly warrants further investigation.

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Chapter 6: Effects of exogenous BDNF on the recovery of muscle and motor unit properties:

A preliminary report

Experiments performed in collaboration with Mr. Neil Tyreman

6.1: Introduction

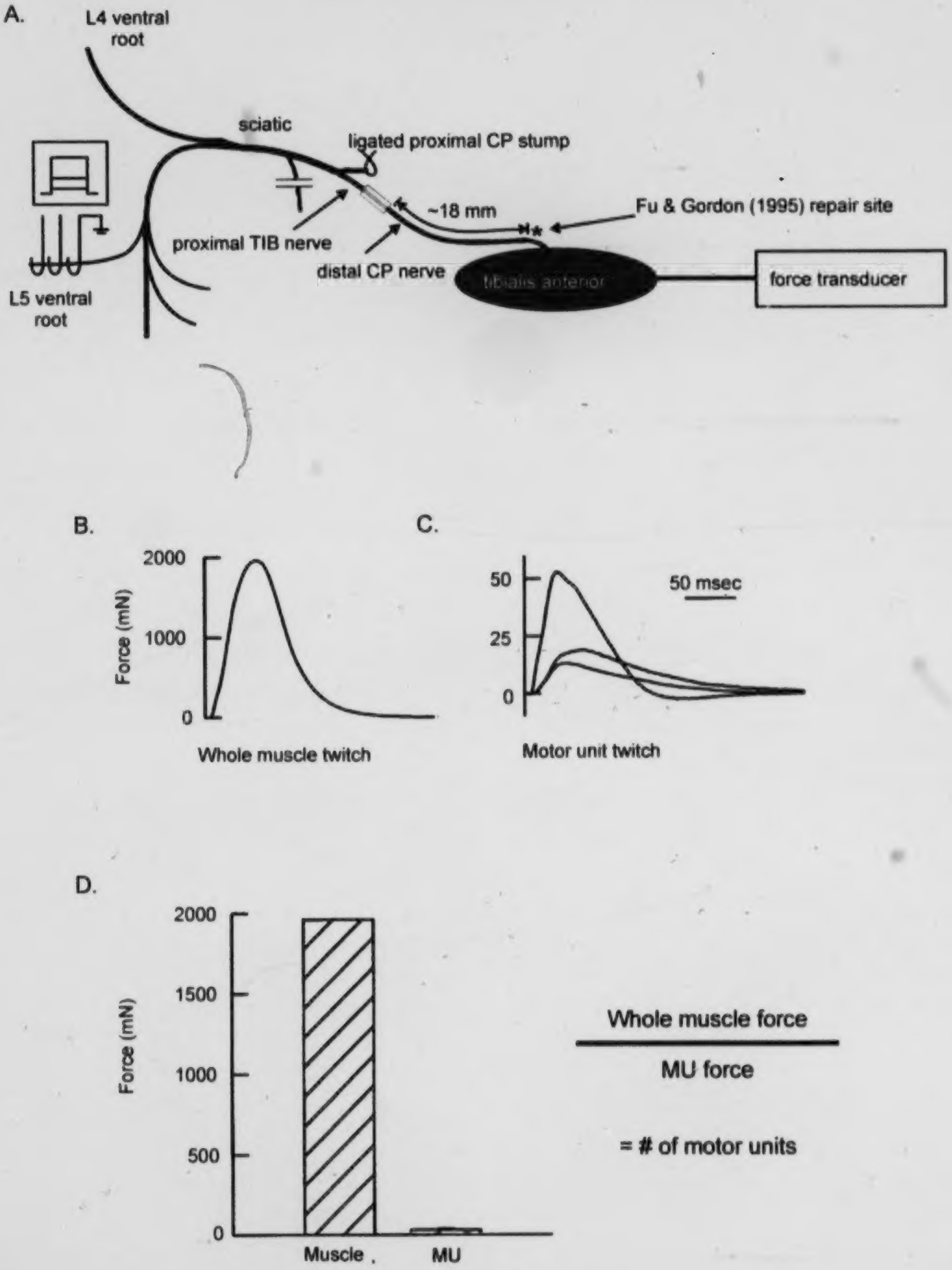
Despite the capacity of peripheral nerves to regenerate their axons after injury, the recovery of function is often poor, and permanent disability may result (Sunderland, 1978; Terzis and Smith, 1990; Lundborg, 2000). Specifically, the restoration of motor function may be quite poor when reinnervation of denervated muscle targets is delayed, due to either the long distance between the target and injury site, or the delayed nerve repair which follows major trauma. For example, following injury to a major nerve plexus (e.g. brachial), peripheral nerve surgeons may wait many weeks to months before attempting any nerve repair in order to properly evaluate the severity of the injury (Lundborg, 2000). This surgical delay, combined with the slow rate of axonal regeneration (1-3 mm/day; reviewed by Seddon, 1975; Bisby, 1995), can lead to periods of over a year before regenerating axons can reach denervated targets. One of the major contributing factors to this poor functional recovery after peripheral nerve injury is that axotomized motoneurons progressively lose their ability to regenerate axons (Chapter 2, 4, 5). This reduced capacity of chronically axotomized motoneurons to regenerate axons correlates well with the time-dependent decline in the number of motor units which reinnervate denervated muscles (Fu & Gordon, 1995).

We have recently demonstrated that continuous administration of low doses of exogenous neurotrophic factors, such as brain derived neurotrophic factor (BDNF; Chapter 2), and glial cell line-derived neurotrophic factor (GDNF; Chapter 4) are effective in completely reversing the negative effects of chronic axotomy on motor axonal regeneration. BDNF belongs to the neurotrophin family of neurotrophic factors which also includes nerve growth factor (NGF), neurotrophin (NT)-3, and NT-4/5 (reviewed in Chapter 1). BDNF mediates its effects by binding to two classes of receptors: a member of the tropomyosin receptor kinase (trk) family of receptors, trkB, and a member of the tumor necrosis family of receptors, p75 (Friedman & Greene, 1999; Yano & Chao, 2000; Chapter 1). In addition to the beneficial effects of low dose BDNF on promoting the axonal regeneration of chronically axotomized motoneurons, progressively higher doses potently inhibit motor axonal regeneration (Chapter 2). Based on experiments using function-blocking antibodies toward p75 (Chapter 2), and either trkB or p75 knockout mice (Chapter 3), the facilitatory and inhibitory effects of BDNF on motor axonal regeneration are attributed to BDNF binding trkB and p75 receptors, respectively. In addition, exogenous GDNF, which does not bind to p75, shows only positive effects on motor axonal regeneration (Chapter 4).

The present study is a preliminary investigation of the effects of both low and high dose exogenous BDNF on muscle reinnervation following nerve repair of chronically axotomized motoneurons to a freshly denervated distal nerve stump; specifically on the number and characteristics of the reinnervated motor units. Using electrophysiological and morphological methods to evaluate the properties of reinnervated muscle, we demonstrate that the ability of low dose exogenous BDNF to increase the numbers of chronically axotomized motoneurons which reinnervate denervated muscles was in accordance with demonstrations that the BDNF increases the number of motoneurons which regenerate axons into the distal nerve stump. Hence the capacity of BDNF to enhance axonal regeneration was translated into enhanced muscle reinnervation after chronic axotomy. However, the inhibitory effect of high dose BDNF on motor axonal regeneration was not associated with a corresponding reduction in the number of reinnervated muscle fibres. Thus, the inhibitory effects of high dose BDNF on motor axonal regeneration are not permanent, and successful reinnervation can occur after the cessation of high dose BDNF treatment.

Figure 6-1: Preparation for final experiment and recording of muscle and motor unit force. *A*. Two month chronically tibial (TIB) axotomized motoneurons were cross-sutured to the common peroneal (CP) distal nerve stump as previously described (Chapter 2) and allowed to regenerate their axons and reinnervate the denervated tibialis anterior muscle for 4 months. All branches of the sciatic nerve were cut, except the TIB, to denervate the hindlimb and hip muscles. The tibialis anterior muscle was isolated and attached to a custom made force transducer. A laminectomy was performed, and the L4 and L5 ventral roots stimulated in a stepwise fashion and the resulting muscle force recorded. *B*. Representative whole muscle twitch force. *C*. Example of 3 motor unit (MU) twitch force responses resulting from stepwise increases in stimulus strength. *D*. The number of MUs was calculated by dividing the average MU twitch force (n=18-46 per animal) by the whole muscle twitch force.

Figure 6-1: Procedures for recording whole muscle and motor unit force


A.
L4 ventral root
sciatic
ligated proximal CP stump
~18 mm
Fu & Gordon (1995) repair site
proximal TIB nerve
distal CP nerve
tibialis anterior
force transducer
L5 ventral root
B.
2000
1000
0
Force (mN)
Whole muscle twitch
C.
50
25
0
50 msec
Motor unit twitch
D.
2000
1500
1000
500
0
Force (mN)
Muscle
MU
Whole muscle force
MU force
= # of motor units

6.2: Methods

6.2.1: Nerve repair and pump implantation

Chronic tibial (TIB) axotomy, the TIB-common peroneal (CP) nerve cross-suture, and pump implantation were performed as described in Chapter 2.

6.2.2: Delivery of exogenous BDNF via Alzet mini osmotic pumps

BDNF (kindly provided by Regeneron Pharmaceuticals) was diluted in saline (0.9% NaCl) and administered via Alzet 28 day mini-osmotic pumps (model 2ML4) in low (2 μg/day) and high (20 μg/day) doses at a continual flow rate of 2.5 μl per hour. Control animals received saline alone. Following the 28 day treatment with saline, low or high dose BDNF, the rats were anaesthetized with sodium pentobarbitol (30 mg/kg) and the mini-osmotic pumps were removed without trauma to the nerve repair site.

6.2.3: Surgical preparation for the final experiment

Four months after TIB-CP cross-suture, and 3 months after cessation of delivery of exogenous BDNF, the tibialis anterior (TA) muscle was isolated in a final experiment for muscle and motor unit (MU) force recordings as previously described (Fu & Gordon, 1995). Briefly, rats were anaesthetized with sodium pentobarbitol (30 mg/kg) and maintained by intravenous injection of 5% of the original sodium pentobarbitol dose diluted in 20% in 5% dextrose-saline via a cannula inserted into the right external jugular vein. Atropine sulphate (0.1 mg/kg) was injected to reduce tracheal secretion. The trachea was cannulated for mechanical ventilation when necessary. Blood volume was maintained by hourly injection of 0.5-1 mL of the 5% dextrose-saline solution via the intravenous cannula.

All hindlimb, hip, and tail muscles innervated by L4-L6 ventral roots were denervated except the TA (Figure 6-1A). Both left and right TA muscles were isolated with their distal tendons tied to small pieces of 0-0 silk suture for later attachment to force transducers. The bared ends of two Teflon-coated fine silver wires, used as stimulating electrodes, were inserted into the muscle beneath the sciatic nerve (proximal to the site of TIB-CP cross suture) for eliciting TA muscle contractions. Both hindlimbs were immobilized at the knees and ankles. The distal TA tendon was attached to a custom-made force transducer for isometric recording muscle and MU forces (figure 6-1A). Muscle and core temperature were maintained with a heating pad and overhead electric bulb.

A laminectomy was performed from T13 to L6. The dura mater was cut. The L4-L6 ventral roots were isolated with a glass rod and gentle suction using a glass pipette (Figure 6-1A). A mineral oil pool was prepared for the spinal cord and the exposed ventral roots by stretching the skin flaps around the incision. Stimulating electrodes for ventral roots were placed above the spinal cord in the mineral oil pool.

6.2.4: Muscle and MU force recordings

Force signals were amplified, monitored on a Tektronix dual time base oscilloscope (model 5441), recorded continuously on a Gould 1200S pen recorder, and digitized and stored with an LSI-11 computer.

The TA muscle was adjusted to its optimal length for recording maximum isometric twitch (Figure 6-1B) and tetanic forces in response to suprethreshold stimulation of the sciatic nerve. Twitch and tetanic contractions were evoked by single stimulus and tetanic trains of 5 and 21 pulses at 100 Hz. Fine filaments containing 3 to 7 axons in the ventral roots L4 and L5 contributing to the TIB nerve were stimulated to elicit all-or-none incremental force responses (Figure 6-1C), and the twitch response of each MU was obtained by digital subtraction. From 20-50 MUs in each reinnervated TA muscle were sampled, which represented at least 50% (and up to 100%) of the total MU population in each muscle.

6.2.5: Muscle fibre histochemistry

After completion of the whole muscle and MU force recordings, the TA muscle was quickly removed, weighed, and cut into three blocks, rapidly frozen in isopentane cooled with liquid nitrogen, and stored in a freezer at -70°C. Serial cross sections (8 μm thick) were then cut and stained for myofibrillar ATPase after acid preincubation according to Green et al. (1982). Muscle fibres were classified into slow oxidative (SO), fast oxidative glycolytic (FOG), and fast glycolytic (FG) as previously described (Tötösy de Zepetnek et al., 1992).

6.2.6: Data Analysis

6.2.6.1: MU number:

At least 50% of the total MU population in each muscle was sampled to obtain a representative mean of MU twitch forces. The total number of MUs in each muscle was calculated by dividing the whole muscle twitch force by the mean MU twitch force (Figure 6-1D; Jansen and Fladby, 1990; McComas, 1991; Fu & Gordon, 1995)

6.2.6.2: Muscle fibre size and number:

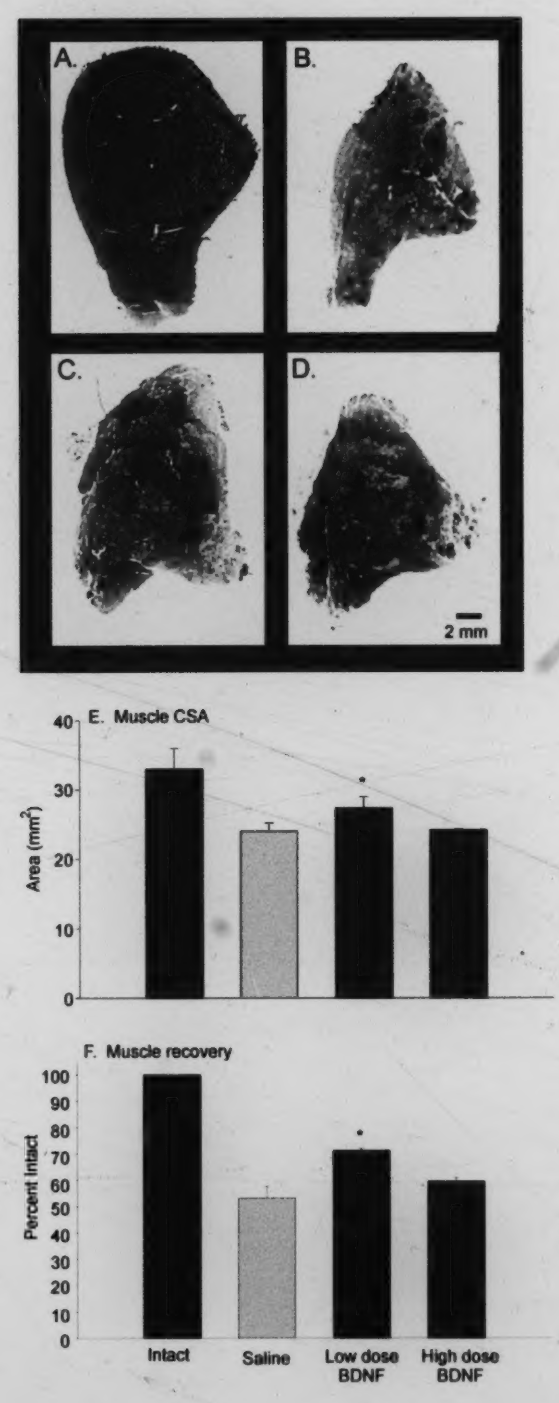
Light microscopic images of the acid ATPase stained 8 µm cross sections of the TA muscle were captured using a Sony LCD camera, and analyzed using JAVA video analysis software (Jandel Scientific, Corte Madera, CA). Whole muscle and muscle fibre cross sectional area (CSA) was measured on cross sections that contained the maximum number of muscle fibers (i.e. the centre of the muscle belly). In the same cross sections, muscle fibers were counted in 0.63 mm^2 areas located in the same comparable 12-15 regions in all the muscles examined. The counting area included at least 25% of the total number of muscle fibres in each muscle. The number of muscle fibres in each muscle was determined by dividing the average muscle fibre CSA by the whole muscle CSA.

6.2.6.3: Statistics

As described in Chapter 2.

Figure 6-2: Low dose BDNF improves recovery of tibialis anterior (TA) muscle cross sectional area (CSA) and muscle mass. *A-D*. Low power photomicrographs of representative 8 μm cross sections of the intact TA muscle (*A*), and reinnervated TA muscle following saline (*B*), low dose BDNF (2 μg/day; *C*), and high dose BDNF (20 μg/day; *D*). *E*. Compared to the intact TA, whole muscle CSA is significantly reduced in reinnervated muscle. Reinnervated TA CSA was significantly larger following low dose exogenous BDNF treatment (n=4) compared to either saline (n=3) or high dose BDNF (n=2) treatment. *F*. The decreased muscle mass in reinnervated TA compared to intact TA is significantly attenuated following low dose exogenous BDNF treatment.

Figure 6.2: Low dose BDNF improves TA recovery



6.3: Results

6.3.1: Whole muscle properties of the reinnervated TA

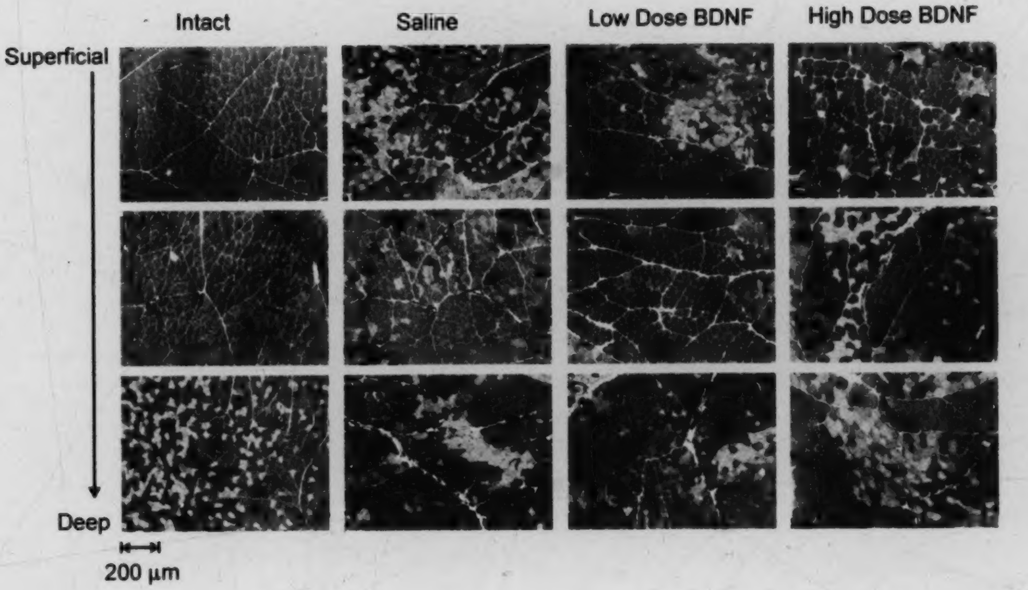
Contralateral intact and reinnervated TA muscles were weighed and evaluated for whole muscle CSA. As shown in the representative whole mount micrographs in Figure 6-2, compared to contralateral intact TA muscles (Figure 6-2A), TA muscles reinnervated by 2 month chronically axotomized TIB motoneurons 4 months after TIB-CP cross-suture and saline treatment (Figure 6-2B) are much smaller. This was consistent with a significant reduction in whole muscle CSA (Figure 6-2E) and muscle mass (Figure 6-2F). The lower 53±4.4% recovery in reinnervated muscle mass compared to contralateral intact control TA muscles was much more dramatic than the 87% recovery previously reported for the same period of chronic axotomy, but not axonal regeneration (Fu & Gordon, 1995). This difference may be explained by the fact that the methods used in this study differ from the methods used in the original paper by Fu & Gordon (1995) in two key ways. First, our site of TIB cross suture was approximately 15-18 mm proximal to the site of nerve repair used by Fu & Gordon (1995; see Figure 6-1A, Fu & Gordon site of TIB-CP cross suture is denoted by asterisk). Secondly, in contrast to the average period of axonal regeneration and recovery allowed by Fu & Gordon (1995) which was approximately 1 year, only a 4 month period of recovery was used in this study.

6.3.2: Low, but not high dose BDNF improve whole muscle properties of the reinnervated TA compared to saline controls

We have previously reported that continuous administration of low dose exogenous BDNF (2 μg/day) significantly increases the number of chronically axotomized TIB motoneurons which regenerate their axons into a freshly cut distal CP nerve stump, completely reversing the negative effects of chronic axotomy (Chapter 2). To determine whether this increase in motor axonal regeneration correlates with increased muscle recovery, we allowed an additional 3 month period of axonal regeneration following cessation of exogenous BDNF treatment for a total of 4 months regeneration. Low dose exogenous BDNF significantly increased both whole muscle CSA (Figure 6-2E) and recovery of muscle mass (Figure 6-2F) compared to saline controls, which is evident in a

Figure 6-3: Changes in muscle fibre size and distribution in reinnervated TA muscles. Representative photomicrographs of 8 μm longitudinal cross sections of the intact (left column) and reinnervated TA muscles.

Figure 6-3: Muscle fibre types in intact and reinnervated TA following saline, low, or high dose BDNF treatment


Intact
Saline
Low Dose BDNF
High Dose BDNF
Superficial
Deep
200 µm

representative whole muscle photomicrograph shown in Figure 6-2C.

One month continuous high dose (20 μg/day) exogenous BDNF treatment has been previously shown to exert potent inhibitory effects on the number of chronically axotomized TIB motoneurons which regenerate their axons in the same 1 month period (Chapter 2). To determine whether this inhibitory effect on motor axonal regeneration translated to reduced TA muscle recovery, axonal regeneration and TA reinnervation were allowed to proceed for an additional 3 months after cessation of high dose exogenous BDNF treatment. Compared to saline controls, there was no significant difference in either the whole muscle CSA (Figure 6-2E) or muscle recovery (Figure 6-2F) with high dose BDNF treatment. The similarity in CSA between high dose BDNF-treated, and saline control muscles is evident in representative photomicrographs (Figure 6, cf. B,D). Thus, in contrast to low dose BDNF, high dose BDNF does not have any detectable beneficial effect on either whole muscle CSA or recovery of reinnervated TA muscles. Moreover, the dramatic reduction in axonal regeneration after 1 month does not translate at 3 months into reduced muscle reinnervation.

6.3.3: Effects of exogenous BDNF on type, size, and number of reinnervated muscle fibres

As shown in Figure 6-3, contralateral intact TA muscles show a distinct segregation of muscle fibre distribution, with the superficial layers consisting of primarily FG muscle fibres, and a mixture of muscle fibre types in the deeper layers, consistent with previous reports (Tötösy de Zepetnek et al., 1992). In reinnervated TA muscles, the fibre type distribution is much more random, and "clumping" of muscle fibres of the same type is readily apparent as reported previously (Fu & Gordon, 1995; Tötösy de Zepetnek et al., 1992). As shown in Figure 6-4, there is a no significant difference in the relative number of FG (Figure 6-4A), FOG (Figure 6-4B), or SO (Figure 6-4C) muscle fibre types.

As shown in 6-5A, in comparison to the muscle fibres in the intact TA muscle, in which the FG muscle fibres are significantly larger than either the FOG or SO fibres, the differences in muscle fibre CSA between different fibre types are much less in reinnervated muscle. This "equalization" of fibre CSA among fibre types is consistent with other studies of reinnervated rat TA (Tötösy de Zepetnek et al., 1992) as well as cat medial gastrocnemius

Figure 6-4: Relative contribution of muscle fibre types in intact (black bars, n=3), and reinnervated TA muscles following saline (grey bars, n=3), low dose BDNF (2 µg/day; blue bars, n=4), or high dose (20 µg/day; red bars, n=2) treatment. There is no significant difference in the relative proportion of FG (*A*), FOG (*B*), or SO (*C*) muscle fibres between all groups examined.

Figure 6-4: Neither reinnervation nor exogenous BDNF significantly change the relative proportion of TA muscle fibre types

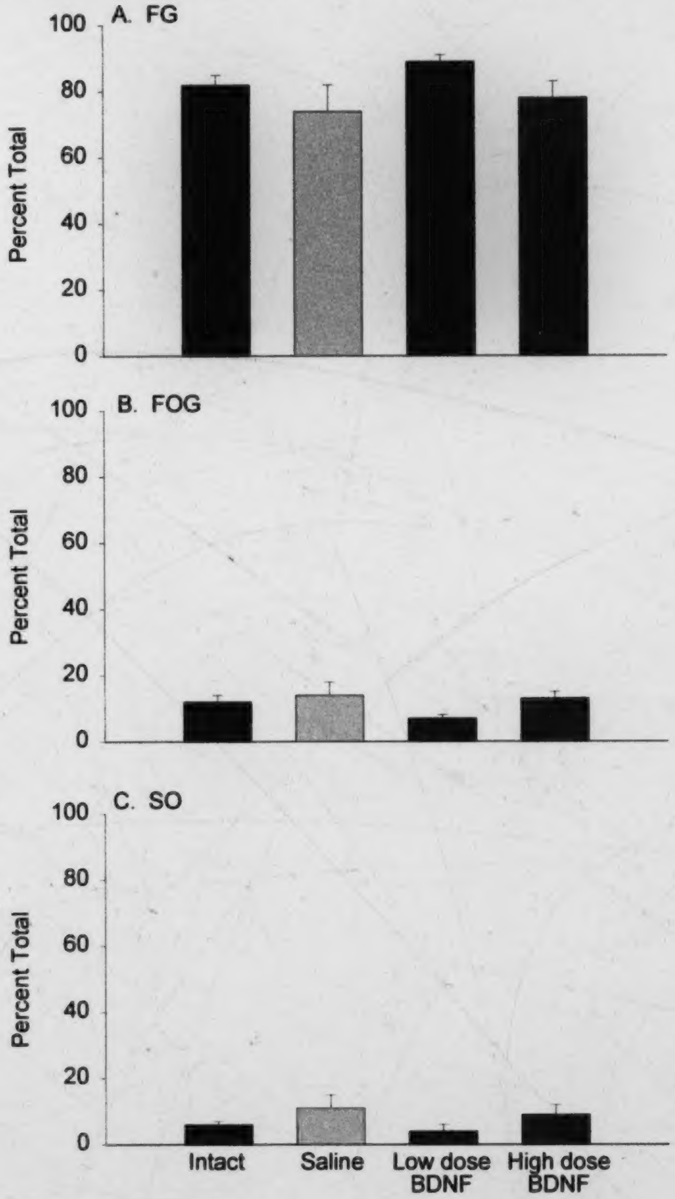
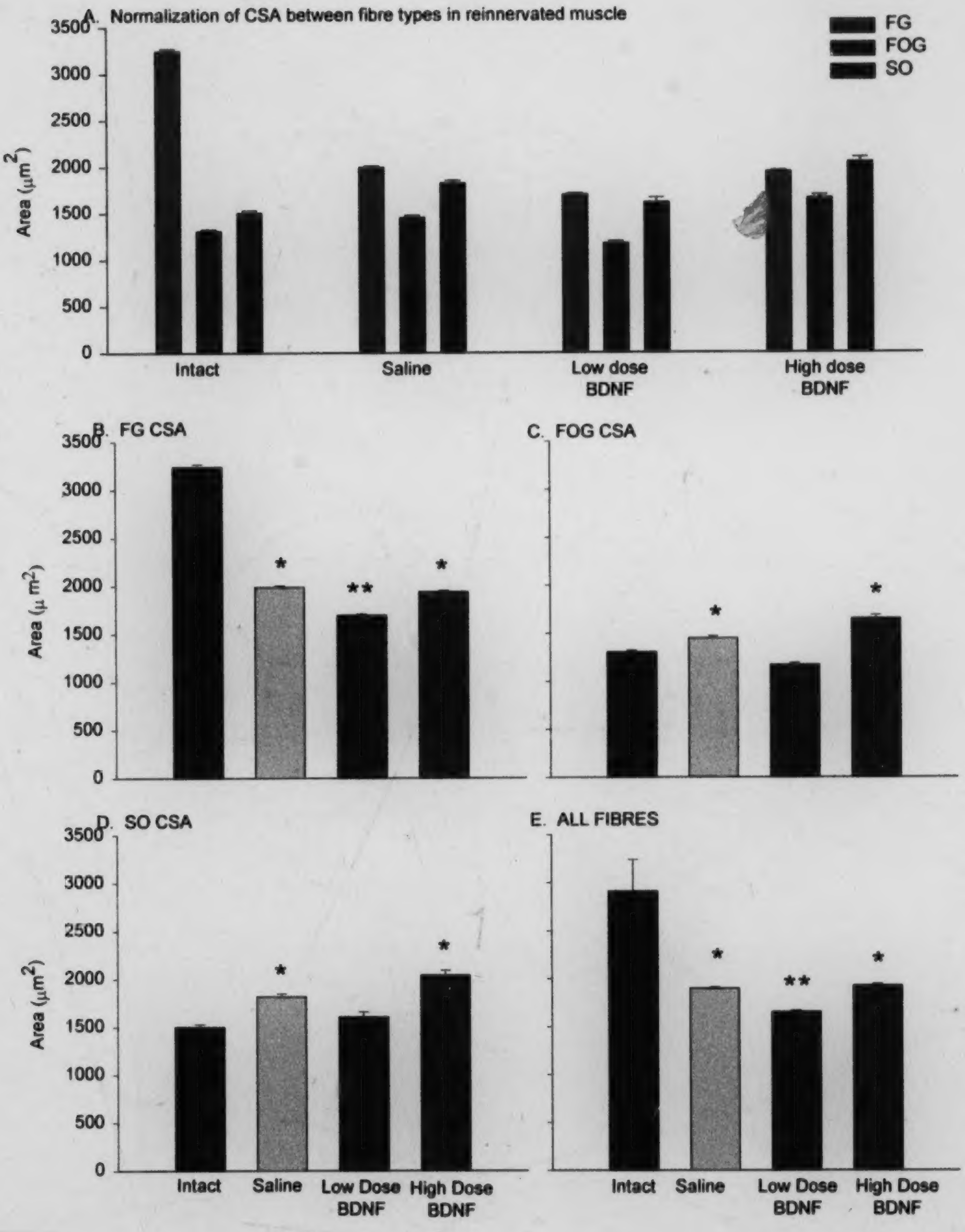


Figure 6-5: Changes in muscle fibre cross sectional area (CSA) following reinnervation and exogenous BDNF treatment. *A*. Muscle fibres of different types are similar in size in reinnervated muscle compared to intact controls, consistent with previous reports. *B-D*. Histograms showing the relative changes in muscle fibre CSA for FG (*B*), FOG (*B*), and SO (*D*) muscle fibre types. *E*. Summary histogram. * denotes significance compared to intact controls, ** denotes significance between treatment groups.

Figure 6-5: Low dose exogenous BDNF reduces muscle fibre CSA

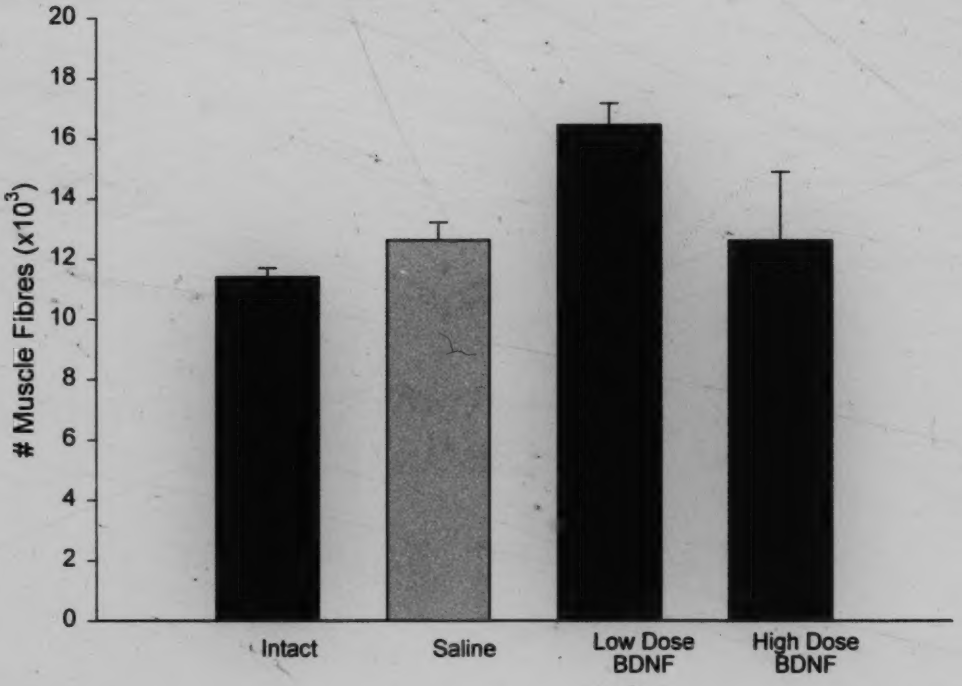


(Rafuse & Gordon, 1996) muscles. Compared to contralateral control TA muscles, there was a significant reduction in FG muscle fibre CSA in all reinnervated TA muscles (Figure 6-5B). This effect was most dramatic following low dose BDNF treatment, with FG muscle fibre CSA significantly lower than in either saline or high dose BDNF treated groups. In contrast, FOG (Figure 6-5C) and SO (Figure 6-5D) muscle fibre CSA is increased in saline and high, but not low dose BDNF treated groups compared to contralateral intact control fibres. The relative changes in fibre CSA are apparent in the representative photomicrographs of 8 μm cross sections of the intact and reinnervated muscles shown in Figure 6-3. Overall muscle fibre CSA was calculated by pooling all muscle fibres measured regardless of fibre type. Due to the high relative proportion of FG muscle fibres (Figure 6-4), overall muscle fibre CSA (Figure 6-5E) follows the same pattern as demonstrated for FG muscle fibres (Figure 6-5B). Again, reinnervated muscle fibres were dramatically smaller than those in intact TA muscles, and this effect is significantly greater in low dose BDNF treated animals. This is in contrast to previous reports of similar fibre CSA between intact and reinnervated TA muscles (Fu & Gordon, 1995), but as with whole muscle CSA, this discrepancy may be due to shorter periods of regeneration, and longer regeneration distances used in this study.

The number of muscle fibres in each TA muscle was calculated by dividing the whole muscle CSA by the average fibre CSA for each individual animal. As shown in Figure 6-6, although there is no difference in the number of muscle fibres in intact, saline- or high dose BDNF treated animals, the significantly larger whole muscle CSA (Figure 6-2) combined with the significantly lower muscle fibre CSA in low dose treated animals can be explained by a greater number of muscle fibres in the TA muscle following low dose BDNF treatment.

Figure 6-6: Number of muscle fibres in intact and reinnervated muscles. The number of muscle fibres was calculated by dividing the whole muscle cross sectional area by the mean fibre cross sectional area. There are significantly more muscle fibres in the TA muscle following low dose BDNF treatment (n=4) compared to intact (n=3), saline (n=3), or high dose BDNF (n=2).

Figure 6-6: Low dose exogenous BDNF increases the number of muscle fibres in TA muscle



6.3.4: Effects of exogenous BDNF on muscle and MU force properties

The muscle and MU force properties of 2 month chronically axotomized TIB motoneurons were evaluated 3 months following cessation of one month's treatment with either saline, low dose, or high dose exogenous BDNF (i.e. 4 months total regeneration time). As shown in the representative traces (Figure 6-7A) and in the summary histogram (Figure 6-7B), TA twitch force is significantly reduced in reinnervated muscles compared to intact controls, but there was no significant difference between any of the treatment groups. However, when TA twitch force is normalized to fibre CSA (Figure 6-7C), there was no significant difference between intact and reinnervated TA muscles. Whole muscle tetanic force was significantly reduced in saline treated reinnervated TA muscles compared to contralateral intact controls (Figure 6-7E), apparent in the representative traces (Figure 6-7D). This slight reduction in tetanic force in the reinnervated muscle is consistent with previous reports (Fu & Gordon, 1995). However, as shown in the representative traces of TA tetanic force (Figure 6-7D) and in the summary histogram (Figure 6-7E), there was no significant reduction in the amount of tetanic force generated between intact and reinnervated TA muscles treated with either low or high dose exogenous BDNF. When TA tetanic force is normalized to muscle fibre CSA, there was significantly greater force generated by reinnervated TA muscles following low dose BDNF treatment than in either saline or high dose BDNF treated, or even intact TA muscles (Figure 6-7F). The increased tetanic force produced by reinnervated TA muscles following low dose BDNF treatment could possibly be due to the slight increase in total number of reinnervated muscle fibres (Figure 6-6).

The mean MU force and force distribution was significantly shifted to higher forces in reinnervated TA muscles following saline treatment compared to intact TA MUs (Figure 6-8). Exogenous BDNF significantly shifted the mean MU force and the distribution of TA motor unit force towards intact force levels, but this effect was not dose-dependent.

Figure 6-7: Effects of reinnervation and exogenous BDNF treatment on whole muscle properties of TA. *A*. Representative traces of TA twitch force in intact (black line), and reinnervated muscle following saline (grey line), low dose BDNF (blue line), and high dose BDNF (red line) treatment. The low and high dose BDNF traces are overlapping. *B*. Compared to intact controls, there was a significant reduction in whole muscle twitch force in reinnervated muscles, regardless of treatment group. *C*. After normalizing the whole muscle twitch force to muscle fibre cross sectional area (CSA), there is no significant difference between intact and reinnervated muscles, regardless of treatment group. *D*. Representative trances of TA tetanic force in intact (black line), and reinnervated muscle following saline (grey line), low dose BDNF (blue line), and high dose BDNF (red line) treatment. *E*. Histogram showing the reduction in whole muscle tetanic force in saline treated, but not exogenous BDNF treated groups. *F*. After normalizing the whole muscle tetanic force to muscle fibre CSA, significantly greater force is generated in reinnervated muscle following low dose BDNF, but not saline or high dose BDNF treatment.

Figure 6-7: Effects of exogenous BDNF on whole muscle properties of TA

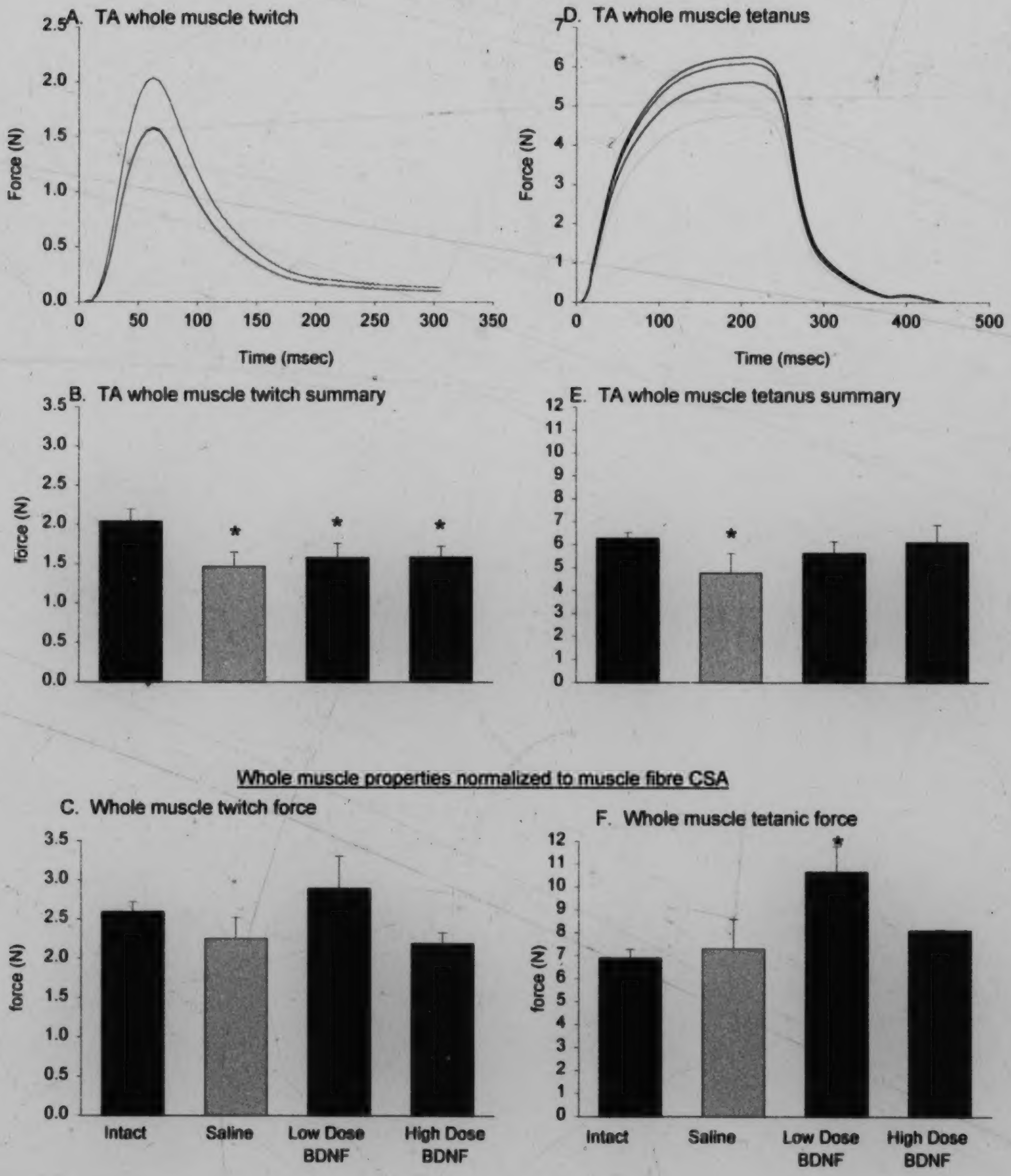
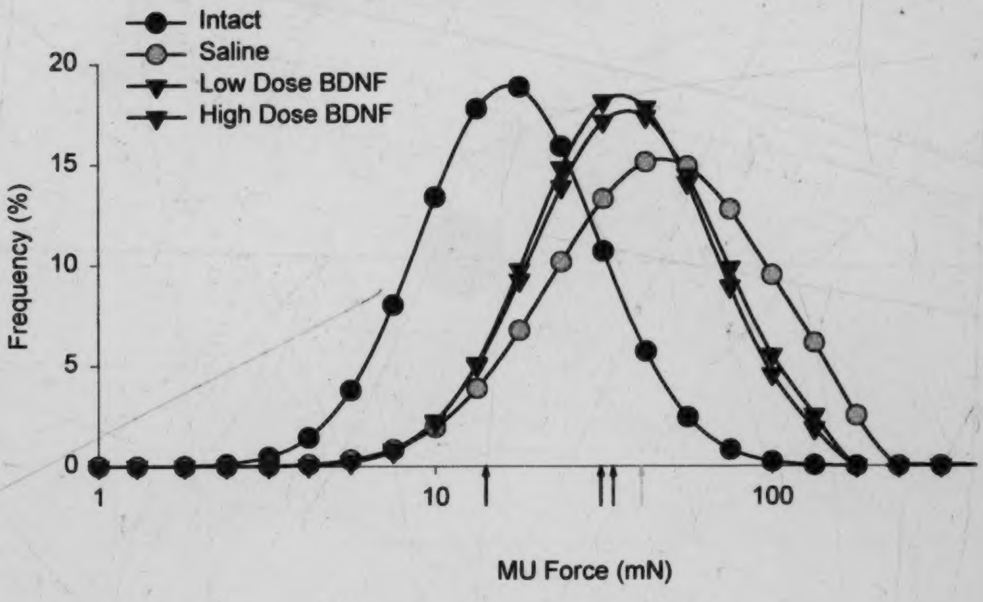


Figure 6-8: Frequency distribution of motor unit (MU) force in intact and reinnervated TA muscle. Compared to intact controls (black line), distribution of MU force is shifted to higher forces in reinnervated muscles (mean MU force is shown as arrows on X-axis). MU force is normalized in BDNF treatment groups, but this effect is not dose-dependent.

Figure 6-8: Frequency distribution of TA motor unit force is shifted towards intact in BDNF treated animals compared to saline controls

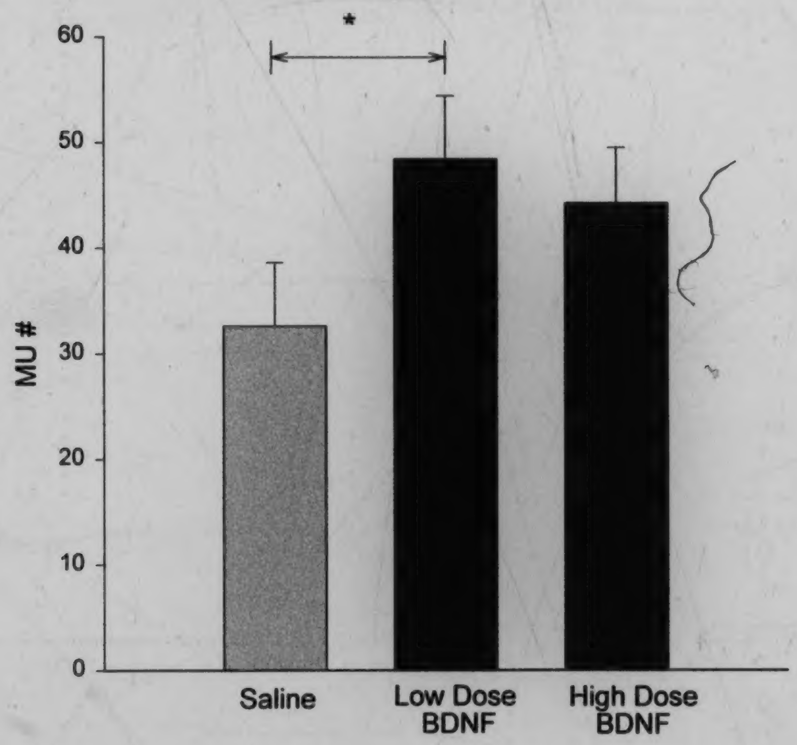


6.3.5: Low, but not high dose BDNF increases the number of MUs innervating the denervated TA muscle.

The number of MUs reinnervating the denervated TA muscle was calculated by dividing the whole muscle twitch force by the average MU twitch force as previously described (Jansen & Fladby, 1990; McComas, 1991; Fu & Gordon, 1995). A mean (±SE) number of 32.6 ± 6.2 MUs reinnervated the TA muscle following a 2 month period of chronic axotomy prior to nerve repair and a 4 month period of regeneration (Figure 6-9). This represents only ~25% of the total number of MUs which normally innervated the TA muscle (Fu & Gordon, 1995). This was much lower than the ~69% of 2 month chronically axotomized motoneurons which regenerated their axons a distance of 20 mm in a one month period of time
(Chapter 2), and the ~66% of MUs which reinnervated the TA muscle following the same 2 month period of chronic axotomy (Fu & Gordon, 1995). One month continuous treatment with low dose exogenous BDNF showed a slight, but significant increase in the number of MUs innervating the TA muscle, an effect which was not significant with high dose BDNF (Figure 6-9). The beneficial effect of low dose BDNF represents a 140% increase in the number of MUs innervating the TA muscle compared to saline controls. This increase was less dramatic than the 182% increase in the number of chronically axotomized motoneurons which regenerated their axons in a one month period of time (Chapter 2). Thus it is possible that sustained administration of exogenous neurotrophic factors such as BDNF is required for maximal beneficial effects of increasing the number of motoneurons reinnervating the denervated TA. This would be consistent with our previous study which demonstrated that continuous, rather than short-term exogenous neurotrophic factor treatment is necessary for maximal beneficial effect on motor axonal regeneration (Chapter 4).

Figure 6-9: Number of motor units (MUs) in reinnervated muscle. The number of MUs was calculated by dividing the whole muscle twitch force (see Figure 6-7) by the mean MU twitch force (see Figure 6-8). Compared to saline controls (grey bar), there is a significant increase in the number of MUs following low dose BDNF (blue bar), but not high dose BDNF (red bar) treatment.

Figure 6-9: Low dose exogenous BDNF increases the number of chronically axotomized TIB motor units which reinnervate the denervated TA muscle



6.4: Discussion

The present study was a preliminary investigation using direct and quantitative methods to evaluate the effects of exogenous BDNF on functional motor recovery in a condition whereby motoneuronal regenerative capacity is reduced by chronic axotomy. Our results demonstrate that continuous administration of low doses of BDNF previously reported to promote axonal regeneration of chronically axotomized motoneurons (Chapter 2) increases the number of MUs reinnervating the TA muscle (Figure 6-9) which was associated with an improvement in several whole muscle properties including CSA (Figure 6-2), recovery in mass (Figure 6-2), and tetanic force (Figure 6-7). The profound inhibitory effects of high dose BDNF on motor axonal regeneration (Chapter 2) did not directly translate to a functional deficit compared to saline controls. In fact, the effects of high dose BDNF were similar to saline controls on nearly all parameters examined, with two important exceptions: compared with saline controls, high dose exogenous BDNF 1) restored the recovery of whole muscle tetanic force (Figure 6-7), and 2) normalized the MU force distribution (Figure 6-8).

6.4.1: Low dose BDNF improves functional recovery

There is substantial evidence to suggest an important role of BDNF promoting recovery of motor function after motor axonal injury: 1) low dose BDNF increases motor axonal regeneration of chronically axotomized motoneurons, 2) BDNF promotes both motoneuronal neurite outgrowth *in vitro* (Dolcet et al., 1999; Atwal et al., 2000), and motor axonal outgrowth after ventral root avulsion (Novikova et al., 1997; Novikov et al., 1997; Kishino et al., 1997), 3) accelerated femoral motor axonal regeneration induced in response to short term electrical stimulation (Al-Majed et al., 2000a) is correlated with accelerated temporal kinetics of BDNF and trkB mRNA expression (Al-Majed et al., 2000b). Therefore, findings that exogenous BDNF does not enhance sciatic functional recovery as evaluated by sciatic function index (Moir et al., 2000), gait analysis (Shirley et al., 1996), or force recovery (Shirley et al., 1996) are quite surprising. However, the indirect methods of evaluating functional recovery, such as the sciatic functional index (a calculated score from walking track analysis), make these results difficult to interpret. For example, Fu & Gordon (1995) demonstrated that despite a 66% reduction in the number of MUs innervating the TA muscle after long term periods of chronic axotomy, there were no observable changes in whole muscle twitch or tetanic forces. These data highlight the extensive axonal branching ability of axotomized motoneurons which functionally compensate for the reduction in motor axons which reinnervate denervated muscle. These data also demonstrate the difficulty in interpreting functional data, such as walking track analysis or force measurements alone.

We have demonstrated that 4 months after delayed nerve repair of chronically axotomized motoneurons to a freshly denervated distal nerve stump, only 25% of the total number of intact TIB motoneurons successfully reinnervated denervated TA muscle fibres. This low number of MUs is less than half the number of MUs reported by Fu & Gordon (1995) after a 12 month period of regeneration. In light of the excellent agreement between the number of chronically motoneurons which regenerated their axons a distance of 20 mm (69%) in one month (Chapter 2), and the number of 2 month chronically axotomized motoneurons which reinnervated the denervated TA muscle in 12 months (66%; Fu & Gordon, 1995), one might anticipate that the number of MUs in the reinnervated TA muscle would increase over a protracted period of time. This gradual increase in the number of motoneurons which reinnervate the denervated muscle fibres would be consistent with the prolonged period of time it takes for motoneurons to regenerate their axons across the injury site and into the distal nerve stump (Al-Majed et al., 2000a).

The ability of low dose BDNF to increase the number of MUs which reinnervate the denervated TA muscle correlates well with an increase in motor axonal regeneration (Chapter 2), whole muscle CSA (Figure 6-2), recovery in mass (Figure 6-2), and tetanic force (Figure 6-7). However, these beneficial effects of low dose BDNF are not associated with an increase in muscle fibre CSA (Figure 6-5). In fact, low dose BDNF was surprisingly associated with decreased muscle fibre CSA, which in light of the increase in whole muscle CSA indicates a greater number of innervated muscle fibres than in even intact control muscles (Figure 6-6). These data imply a possible novel myogenic effect of low dose exogenous BDNF. The ability of exogenous BDNF to induce myogenesis would be consistent with the well documented myogenic ability of many other small peptide growth factors, such as insulin-like growth factors, fibroblast growth factors, neuregulins, and members of the transforming growth factor-β family (reviewed in Ohuchi & Noji, 1999;

Florini et al., 1996; Husmann et al., 1996; Magri et al., 1991). In addition, a recent study by Peroulakis & Forger (2000) demonstrates that exogenous application of ciliary neurotrophic factor, a neurotrophic factor which shows overlapping effects with BDNF on axotomized motoneurons (Chapter 1), increases the number of muscle fibres in the levator ani muscles in postnatal rats by 300%. Furthermore, myocyte differentiation and proliferation are controlled via the activation of the ras-erk pathway (reviewed in Naya & Olson, 1999), which is activated in response to BDNF binding to trkB receptors (Chapter 1; Dolcet et al., 1999; Atwal et al., 2000). Finally, skeletal muscle expresses the receptors for BDNF, trkB and p75 (Wheeler & Bothwell, 1992; Gonzalez et al., 1999). The ~50% more reinnervated muscle fibres in the TA muscle following low dose exogenous BDNF treatment compared to intact or saline treated reinnervated muscles (Figure 6-6) detected in this preliminary study clearly warrants further investigation into this putative myogenic effect of low dose BDNF.

It has been recently demonstrated that NT-3 delivered the site of axonal regeneration via fibronectin mats which bridged a 10 mm gap between proximal and distal sciatic nerve stumps significantly increased the number of axons which crossed the gap and penetrated the distal nerve stump compared to fibronectin mats alone (Sterne et al., 1997a). This increase in axon number was associated with a selective reinnervation of FG muscle fibres (Sterne et al., 1997b). The ability of NT-3 to promote selective reinnervation of fast muscle fibres is supported by the fact that NT-3 increased the number and size of reinnervated neuromuscular junctions in the fast extensor digitorum longus muscle, but had no effect on reinnervation of the slow soleus muscle after sciatic nerve repair (Simon et al., 2000). Although it is an enticing possibility that different neurotrophic factors promote selective innervation of specific muscle fibre types, our preliminary findings do not support this hypothesis, as there was no difference in the relative composition of fibre types in reinnervated muscle fibres regardless of treatment group (Figure 6-4), nor differential effects of exogenous BDNF on individual muscle fibre type CSA (Figure 6-5).

6.4.2: High dose BDNF locally inhibits motor axonal regeneration, but "prepares" motoneurons for regeneration

In contrast to virtually complete inhibitory actions of high dose exogenous BDNF on the axonal regeneration of chronically axotomized motoneurons (<10%; Chapter 2), if axonal regeneration is allowed to proceed for an additional 3 months after cessation of the application of exogenous high dose BDNF, compared to saline controls, no inhibitory effects on the functional recovery of denervated muscles are detectable. Interestingly, the beneficial effects of low, but not high dose exogenous BDNF on MU recovery and muscle reinnervation are comparable to the effects of exogenous BDNF on soma size of chronically axotomized motoneurons (Chapter 2). This further supports the hypothesis that the inhibitory effects of high dose BDNF are mediated by signalling events localized to the regenerating growth cones, such as the p75 mediated generation of ceramide (Chapter 5), or other redundant inhibitory signalling events. The lack of functional deficit following high dose BDNF treatment suggests that not only are the inhibitory actions of high dose exogenous BDNF completely reversible, but it is possible that axonal regeneration is actually accelerated following cessation of treatment. Motor axons which were previously prevented from regenerating actually "catch up", such that there was no significant difference in the number of MUs innervating denervated muscle compared to saline controls 4 months after nerve repair (Figure 6-9).

One of the best characterized effects of high doses (12-60 μg/day) exogenous BDNF on axotomized motoneurons is the upregulation of enzymes associated with cholinergic transmission, such as choline acetyltransferase (ChAT) and acetylcholinesterase (AChE; Friedman et al., 1995; Tuszynski et al., 1996; Fernandez et al., 1998; Kishino et al., 1997, 1998). In addition to being important for nerve-muscle communication, acetylcholine (ACh) may be released from regenerating motor nerve terminals and be important in nerve-Schwann cell communication. *In vitro*, motor axons secrete ACh prior to contact with muscle fibres (Stoop & Poo, 1996), and focal application of ACh can induce waves of Ca^{2+} influx in terminal Schwann cells (Georgiou et al., 1999). Moreover, a critical role of cholinergic transmission in motor axonal regeneration is supported by preliminary findings that continuous local pharmacological blockade of muscarinic and nicotinic ACh receptors significantly reduces motor axonal regeneration (N. Mehra, T. Gordon, unpublished observations).

An increased capacity for cholinergic transmission following high dose exogenous

BDNF treatment is also consistent with our data which show that although high dose BDNF shows no significant beneficial effect on whole muscle CSA or recovery (Figure 6-2), or number of MUs (Figure 6-9), the whole muscle tetanic force was restored to control levels (Figure 6-7), and the MU force distribution is significantly shifted towards intact compared to saline controls (Figure 6-8). Therefore, our data suggest that the fewer motor axons which reach denervated muscle fibres after high dose exogenous BDNF treatment are perhaps better able to form functional neuromuscular junctions and reinnervate the denervated muscle fibres.

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Chapter 7: General Discussion

7.1: Caveats and limitations of experimental model

There are a few caveats and limitations to the experimental models used in this thesis, which although discussed in the context of each chapter, warrant specific address here. One common difficulty in the interpretation of the data presented in this thesis lies in the inability to exclude the possibility that the changes in the number of motoneurons which regenerate their axons, either in response to exogenous neurotrophic factors, or genetic mutation of neurotrophic factor receptors, are a result of an indirect effect on the non neuronal cells of the distal nerve stump. A second caveat is more specific to the experiments described in Chapter 3 using p75 (-/-) and trkB (+/-) knockout mice. Because these receptors are intimately involved in motoneuronal survival during development (see Chapter 3), it is possible that the motoneurons which survive into adulthood in these two strains of knockout mice are different that wild type motoneurons. For example, there are significantly fewer motoneurons in the tibial motoneuron pool in p75 (-/-) mice compared to wild type controls (Figure 3-2), but there is greater motor axonal regeneration after injury in these p75 (-/-) mice. Thus, the possibility exists that the fewer surviving motoneurons in p75 (-/-) mice represent a faster-regenerating subpopulation of motoneurons.

These issues may be resolved in the near future, with recent technological advances in the generation of transgenic and knockout mice. Specifically, tissue specific knockouts, under inducible promoters are allowing investigators to selectively ablate genes in adult animals. Such technology suggests the possibility that we may soon be able to knockout neurotrophic factor receptors, either on the Schwann cells, or neurons, in adult animals which have developed normally. Experiments such as these should provide powerful new insight into the mechanisms involved in peripheral nerve regeneration.

7.2: Therapeutic rationale for using neurotrophic factors to sustain motor axonal regeneration

The experiments described in this thesis characterize the role of neurotrophic factors in motor axonal regeneration. I have demonstrated that chronic axotomy reduces the capacity of motoneurons to regenerate their axons (Chapter 2, Chapter 4, Chapter 5). These results are in agreement with the progressive decline in the number of motoneurons which reinnervate denervated muscle as a function of chronic axotomy (Fu & Gordon, 1995; *see* Chapter 6). Thus the diminished ability of chronically axotomized motoneurons to regenerate their axons over time is one of the major contributing factors to the poor functional recovery following peripheral nerve injury. I have used a quantitative method to extensively characterize the role of two neurotrophic factors in motor axonal regeneration, brain derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF). Continuous administration of low doses of exogenous BDNF (Chapter 2) and GDNF (Chapter 4) are extremely effective in reversing the negative effects of chronic axotomy. The increased number of chronically axotomized motoneurons which regenerated their axons with low dose exogenous BDNF is associated with increase in muscle recovery and motor unit reinnervation (Chapter 6). These data provide clear rationale to further investigate the feasibility of using continuous low dose administration of exogenous neurotrophic factors such as BDNF and GDNF to sustain motor axonal regeneration over extended periods of time. Moreover, these experiments have provided a much more clearly defined role for neurotrophic factors in motor axonal regeneration. Specifically, in line with the derivation of the word neurotrophic, which literally means "food for neurons", these experiments demonstrate that neurotrophic factors act to provide sustenance for regenerating motor axons over their long and difficult journeys to reinnervate denervated muscle fibres.

Although the experiments described in this thesis focused on the role of exogenous BDNF and GDNF in motor axonal regeneration, the methods used to quantify their effects on the number of motoneurons which regenerate their axons provide a convenient and effective model to evaluate the therapeutic potential of many neurotrophic factors. For example, many other growth factors have been demonstrated to promote motoneuronal survival and neurite outgrowth, including the insulin-like growth factors, neuropoetic cytokines, and fibroblast growth factors (*see* Chapter 1; Fu & Gordon, 1997), yet their role in motor axonal regeneration awaits definition.

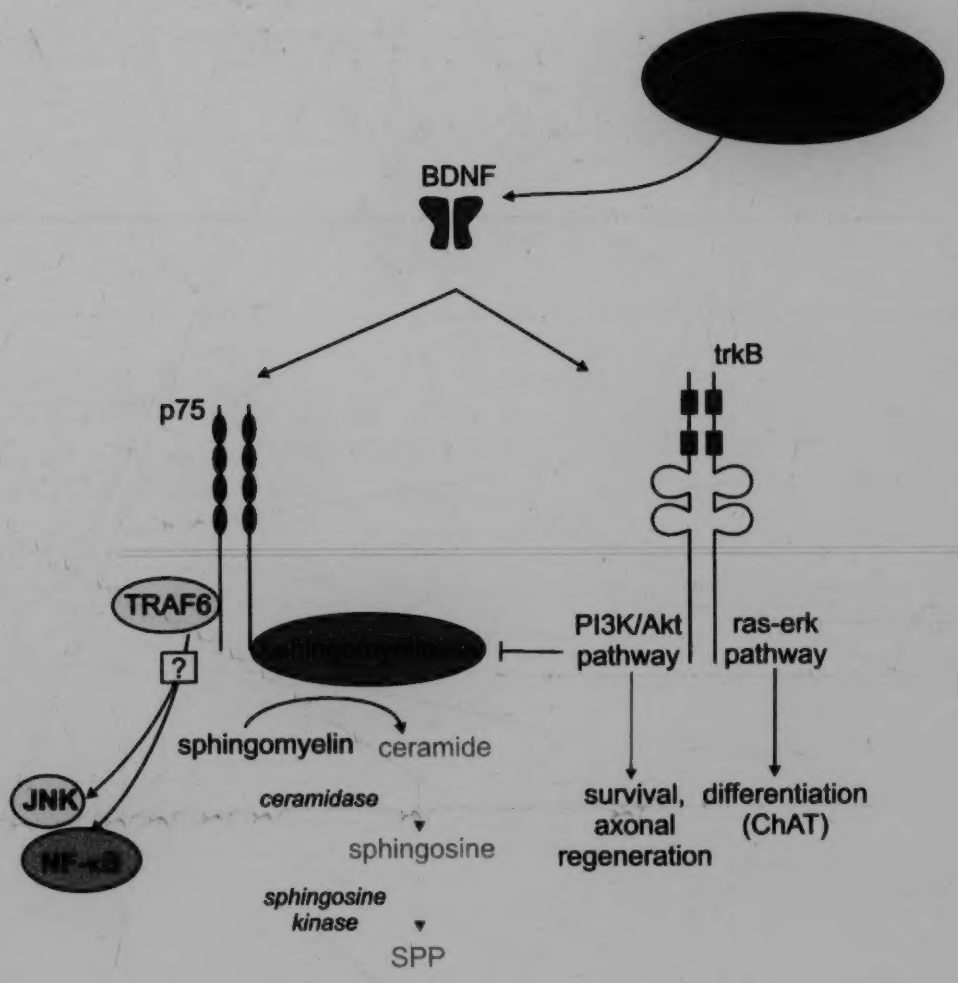
Although exogenous application of neurotrophic factors may effectively reverse the effects of chronic axotomy, there may be other more clinically applicable methods to sustain motor axonal regeneration over time. For example, the short periods (1 hour) continuous electrical stimulation which have been shown to increase the number of motoneurons which regenerate across the injury site and into a distal nerve stump (Al-Majed et al., 2000a), are associated with increased expression of both BDNF and trkB (Al-Majed et al., 2000b) as well as expression of regeneration associated genes (Al-Majed et al., *in revision*). It is an exciting possibility that these short periods of continuous electrical stimulation may also increase the regeneration of chronically axotomized motoneurons. Short periods of electrical stimulation are an enticing clinical possibility, as this procedure can be performed intraoperatively. Furthermore, as most peripheral surgeries on major nerve trunks (e.g. brachial plexus) are performed many weeks to months after the initial injury (*see* Lundborg, 2000), surgeons are already repairing chronically axotomized motoneurons with reduced capacity to regenerate their axons.

7.3: Proposed mechanism for biphasic regulation of motor axonal regeneration by exogenous BDNF

In addition to the beneficial effects of low dose BDNF in promoting motor axonal regeneration of chronically axotomized motoneurons, high dose BDNF dramatically reduces the number of both acutely and chronically axotomized motoneurons which regenerate their axons (Chapter 2). The bimodal facilitatory and inhibitory effects of low and high dose exogenous BDNF are attributed to binding to trkB and p75 receptors, respectively. This hypothesis is supported by several lines of evidence presented in this thesis: 1) the inhibitory effects of high dose exogenous BDNF can be blocked by preventing BDNF binding to p75 receptors with a function-blocking antibody (Chapter 2), 2) there is increased motor axonal regeneration in homozygous p75 knockout mice, and reduced regeneration in heterozygous trkB knockout mice (Chapter 3), 3) GDNF which does not bind p75 receptors, only has

Figure 7-1: Proposed mechanisms of neurotrophic action during the early periods of motor axonal regeneration. Based on *in vitro* data, BDNF (secreted in low levels by denervated Schwann cells) binding to trkB receptors activates the PI3K-Akt pathway, and the ras-erk pathway. Activation of the PI3K pathway promotes motoneuronal survival and axonal regeneration and/or inhibits sphingomyelinase, and thus the production of ceramide (shown in grey).

Figure 7-1: Possible neurotrophic factor interaction at early stages of axonal regeneration


BDNF
p75
trkB
TRAF6
?
sphingomyelinase
PI3K/Akt pathway
ras-erk pathway
sphingomyelin
ceramide
JNK
NF-κB
ceramidase
sphingosine
sphingosine kinase
SPP
survival, axonal regeneration
differentiation (ChAT)

facilitatory effects on motor axonal regeneration, and these effects are not dose-dependent (Chapter 4), and 4) analogs of ceramide, a lipid second messenger downstream of p75 activation, mimics the inhibitory effects of high dose exogenous BDNF on motor axonal regeneration after immediate nerve repair (Chapter 5). Thus motor axonal regeneration appears to depend on a delicate balance between trkB and p75 activation.

The concept of a trk-p75 rheostat is not a novel idea. It has been known for some time that sensory and sympathetic neuronal survival and neurite outgrowth depend on a balance between trkA and p75 *in vitro* and *in vivo* (*see* Chapter 1 and references cited therein). Recent evidence suggests that motoneuronal survival after injury is also regulated by an analogous trkB-p75 equilibrium (Ferri et al., 1998; Alcantara et al., 1997), and our data suggest that motor axonal regeneration is regulated in the same manner.

7.3.1: Mechanisms of axonal regeneration

What are the downstream mechanisms involved in mediating these facilitatory and inhibitory effects on motor axonal regeneration? A proposed mechanism for neurotrophic factor regulation of motor axonal regeneration is shown in Figure 7-1. During the early phases of motor axonal regeneration, motor axon growth cones are exposed to a large number of neurotrophic molecules (*see* Chapter 1), including BDNF, while regenerating through the permissive environment of the distal nerve stump. It is possible that BDNF binding to trkB receptors on motor axonal growth cones promotes motor axonal regeneration through the PI3K-Akt pathway. This is consistent with our results demonstrating the critical dependence of motor axonal regeneration on trkB receptors (Chapter 3). These trkB receptors have been shown to activate both the PI3K pathway and the ras-erk pathway *in vitro* (Dolcet et al., 1999; Atwal et al., 2000). Furthermore, activation of the PI3K-Akt pathway has been demonstrated to be important in neonatal motoneuronal survival, as well as motor axonal regeneration in the adult (Namikawa et al., 2000). In addition to regeneration promoting activity, the PI3K-Akt pathway may also block the inhibitory pathway which is mediated by BDNF binding to p75 receptors. For example, Akt activation in response to NGF binding to trkA, has been demonstrated to reduce sphingomyelinase activity, and thus the production of ceramide (Bilderback et al., 2001). The ras-erk pathway is also activated in response to BDNF binding to trkB (Docet et al., 1999; Atwal et al., 2000). In contrast to the PI3K-Akt pathway, less is known about the role the ras-erk pathway mediates in response to BDNF, as it does not appear to be necessary nor sufficient for motoneuronal survival (Dolcet et al., 1999; Atwal et al., 2000; Namikawa et al., 2000). However, as described in Chapter 1, *in vitro*, the ras-erk pathway is important in neuronal differentiation. For example, the ras-erk pathway has been shown to be involved in PC12 cell differentiation into a neuronal phenotype, such as withdrawal from the cell cycle, expression of neuron-specific markers, and extension of neurites (reviewed in Friedman & Greene, 1999). Thus the possibility exists that activation of the ras-erk pathway is responsible for the regulation of ChAT activity in motoneurons (i.e cholinergic differentiation). The upregulation of ChAT activity is one of the earliest signs of differentiation of neurons into the motoneuronal phenotype during early development, and this ChAT activity can be induced in embryonic motoneurons *in vitro* by neurotrophic factors such as BDNF, GDNF, and ciliary neurotrophic factor (CNTF), which all activate the ras-erk pathway (Zurn et al., 1996, *see* Chapter 1). This model suggests that motor axonal regeneration and ChAT activity are regulated by distinct mechanisms. Differential regulation of ChAT and axonal regeneration is consistent with reports of a BDNF dose-dependent increase in ChAT immunoreactivity in motoneurons following ventral root avulsion (Kishino et al., 1998) across the same range of doses that I have demonstrated a biphasic dose-dependent facilitation and inhibition of motor axonal regeneration (Chapter 2).

7.3.2: Muscle reinnervation

During periods of muscle reinnervation, the regenerative environment changes dramatically. Regenerating motor growth cones come in contact with denervated muscle fibres which express and secrete distinct molecules from the Schwann cells of the distal nerve stump. As shown in Figure 7-2, NGF is secreted by denervated muscle fibres (Funakoshi et al., 1993), but not Schwann cells (Heumann et al., 1987). In addition, both NT-3 and NT-4/5 are expressed in denervated muscle (Griesback et al., 1995). It was first believed that skeletal muscle was also a source of BDNF (Funakoshi et al., 1993), however *in situ* hybridation analysis has demonstrated that the BDNF mRNA isolated from homogenized skeletal muscle actually comes from the Schwann cells of the intra-muscular nerve (Griesback et al., 1995). It is possible, that because axotomized motoneurons do not express trkA, NGF from denervated skeletal muscle binds to p75 receptors, and shifts the trk-p75 balance in favor of a putative inhibitory pathway. However, NGF is also upregulated in the distal nerve stump during the first week of regeneration (Heumann et al., 1987; *see* Chapter 1). In light of experiments which demonstrate the importance of NGF in Schwann cell migration (Anton et al., 1994), this transient upregulation of NGF may serve to promote Schwann cell organization into the linear Bands of Bungner which guide regenerating axons through the injury site and into the distal nerve stump (reviewed in Fu & Gordon, 1997). Additionally, this transient upregulation of NGF may correspond to the initial slow rate of axonal regeneration after injury, as peak regeneration rates do not occur until up to a 3-5 days post-injury (*see* Holmquist et al., 1993). In other words, the early expression of NGF may bind to p75 receptors on regenerating motor axons and possibly mediate a slight inhibitory effect on axonal regeneration, accounting for the delayed regeneration across the injury site during the first week after injury.

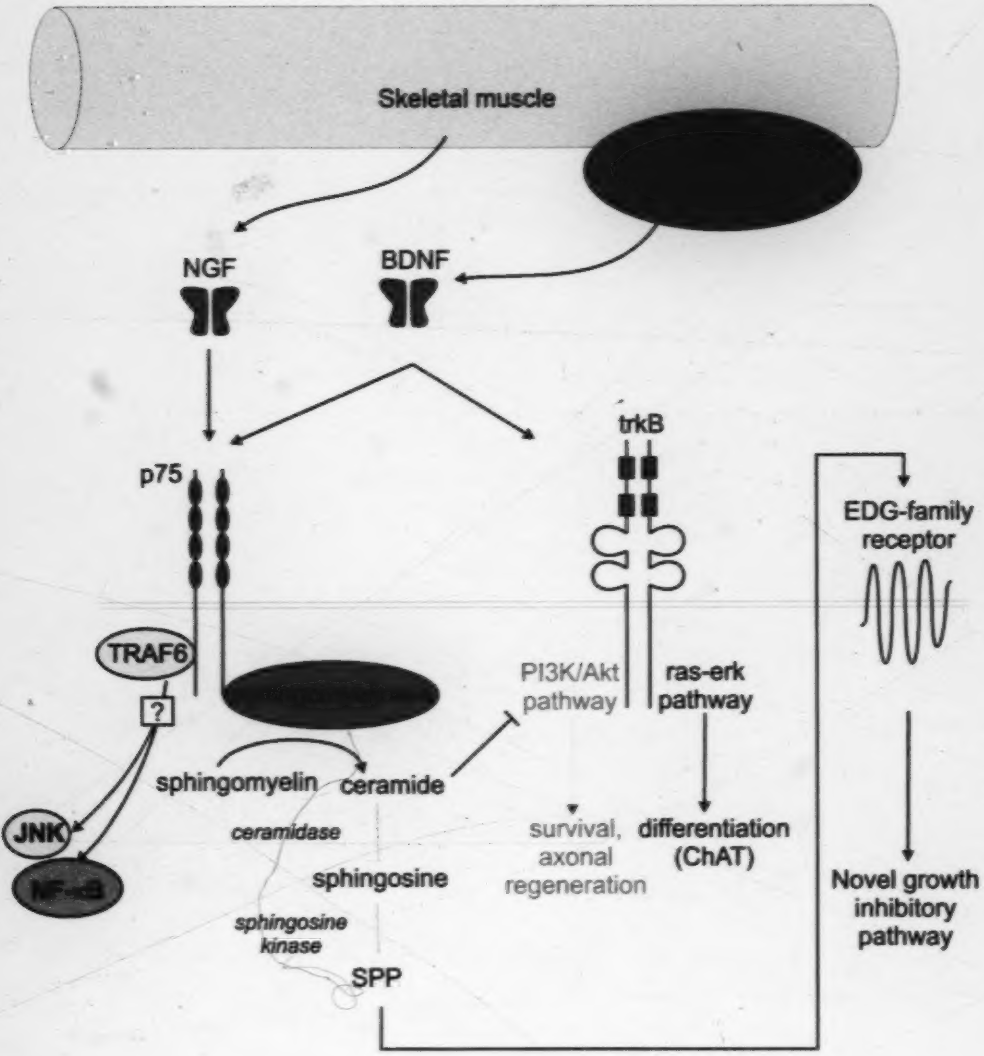
Although the experiments described in this thesis clearly implicate the p75 receptor as the initiator of this inhibitory signal, the exact downstream mechanisms are unclear. Based on the experiments described in Chapter 5, ceramide is a key inhibitory signal, at least after immediate nerve repair. There are several possible mechanisms to explain the inhibitory actions of ceramide (*see* Chapter 5). Ceramide may inhibit trkB receptor activation, as demonstrated for trkA receptors in PC12 cells (MacPhee & Barker, 1997). Ceramide has also been demonstrated to dephosphorylate Akt, thus inactivating the PI3K/Akt pathway (Kim et al., 2001, Zinda et al., 2001, Schubert et al., 2000). The latter possibility would provide a neurotrophic factor-dependent model by which neurotrophins can inhibit axonal regeneration via p75 without affecting motoneuronal regulation of ChAT activity which is maintained by the ras-erk pathway (Figure 7-2). Distinct pathways for regulation of motor axonal regeneration and motoneuronal expression of ChAT is consistent with the fact that doses of BDNF used to upregulate ChAT in axotomized motoneurons (Friedman et al., 1995; Kishino et al., 1997) potently inhibit motor axonal regeneration (Chapter 2).

Another possible mechanism for the p75-mediated inhibition of motor axonal regeneration lies in the metabolism of ceramide. I have demonstrated that sphingosine kinase activity is upregulated in a dose-dependent fashion in response to exogenous BDNF (Chapter 5). This implicates sphingosine-1-phosphate (SPP) in the inhibitory effects of high dose BDNF, but only after immediate nerve repair, and not after chronic axotomy. Interestingly, it has been recently demonstrated that SPP inhibits neurite outgrowth in PC12 cells by binding to a member of the EDG family of G-protein coupled receptors (Figure 7-2; Sato et al., 1997).

Regardless of the downstream mechanisms, it is clear from this data that the p75 receptor signals to inhibit motor axonal regeneration. The possible functional consequence of this inhibitory signal is that NGF derived from skeletal muscle may bind to p75 receptors on regenerating motor axons, and together with other muscle derived signals (e.g. s-laminin) serve as stop signals and initiate the formation of the neuromuscular junction (reviewed in Sanes & Lichtman, 1999). The models presented in Figure 7-1 and Figure 7-2 which describe differential regulation of motor axonal regeneration and muscle reinnervation provide a framework upon which we can critically examine the mechanisms involved in motor axonal regeneration and functional recovery. By being able to selectively activate/inactivate the ras-erk and the PI3K pathways in axotomized motoneurons using adenoviruses (as described in Namakawa et al., 2000), we now have the capacity to fully characterize these pathways and provide direct functional significance to the role of intracellular pathways activated by neurotrophic factors in the regenerating peripheral nervous system. Moreover, with recent identification of other adaptor proteins which associate with p75 receptors, such as TRAF6, NRIF, NRAGE, and SC-1 (reviewed in Kaplan & Miller, 2000), we may be able to further elucidate the mechanisms underlying the inhibitory effect of the p75 receptor on chronically axotomized motoneurons. Finally, in addition to the clinical implications of the beneficial effects of low dose exogenous BDNF on sustaining motor axonal regeneration over extended periods of time, the novel myogenic effect of low dose BDNF suggested by the data presented in Chapter 6 clearly warrants further investigation, as the ability to produce muscle regeneration has large clinical implications for degenerative muscular diseases, such as muscular dystrophy.

Figure 7-2: Proposed mechanisms of neurotrophic action during the late periods periods of motor axonal regeneration (approaching denervated skeletal muscle). NGF (from denervated muscle) binds to p75 receptors on motor axonal growth cones and leads to activation of sphingomyelinase and thus the production of ceramide. Ceramide may either directly inhibit the growth-promoting activities of the PI3K-Akt pathway, or be metabolized into sphingosine-1-phosphate (SPP) and mediate a novel inhibitory effect by binding to EDG-family receptors. The ras-erk pathway remains unaffected, and thus motoneurons are able to upregulate ChAT and initiate neuromuscular cholinergic transmission.

Figure 7-2: Possible neurotrophic factor interaction during muscle reinnervation


Skeletal muscle
NGF
BDNF
trkB
p75
EDG-family receptor
TRAF6
?
PI3K/Akt pathway
ras-erk pathway
sphingomyelin
ceramide
JNK
NF-κB
ceramidase
sphingosine
survival, axonal regeneration
differentiation (ChAT)
Novel growth inhibitory pathway
sphingosine kinase
SPP

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